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20040423

**USE OF THE CANADIAN LIGHT SOURCE TO  
IMPROVE SASKATCHEWAN FEEDS AND CATTLE  
PRODUCTION**

**Funded by: The Agriculture Development Fund**

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**Prepared by: University of Saskatchewan (U of S)**

**FINAL REPORT**



**Use of Canadian Light Source (National Synchrotron Facility) to Understand and Improve  
the Nutritive Value of Plant Materials for Livestock**

**ADF Project # 20040423**

**Final Report**

Milestone Date:

**February, 2010**

By:

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## Table of Contents

|                                                                                                                                                                 |     |
|-----------------------------------------------------------------------------------------------------------------------------------------------------------------|-----|
| Executive Summary.....                                                                                                                                          | 3   |
| 1. Use of SR-FTIR microspectroscopy as a tool for evaluating the<br>degradation characteristics of cereal grains fed to ruminants. ....                         | 6   |
| 2. Study of flaxseed inherent structures affected by heat processing in<br>relation to utilization and availability in ruminants.....                           | 59  |
| 3. Phosphorus Speciation of Dried distiller's Grains with Solubles .....                                                                                        | 74  |
| 4. Use of synchrotron radiation FTIR Microspectroscopy to determine the<br>distribution of cellulosic compounds and lignin components of plant<br>biomass ..... | 81  |
| 5. Appendix A .....                                                                                                                                             | 95  |
| 6. Appendix B .....                                                                                                                                             | 104 |

## **Executive Summary**

The overall objective of this program is to use synchrotron-based analytical techniques to understand how plant structure and composition influences nutritive value and animal performance of cattle and to provide this information to ruminant nutritionists and plant breeders interested in developing superior feeding programs for growing and finishing cattle.

### **Specific objectives include:**

1. To identify features of structural and non-structural carbohydrates, matrixes of biological components and nutrient interactions at cellular and molecular levels to help understand the relationship of complex feed intrinsic structures relative to feed quality, nutrient utilization and digestive behaviour in cattle;
2. To understand the improvement in nutrient utilization of feed materials after feed processing and treatments using synchrotron-based analytical techniques;
3. To increase knowledge of the nutritional relevance of feed chemistry and structure in animal nutrition, for the development of high quality feeding programs through plant breeding and/or feed processing techniques.

To achieve these objectives, 4 trials were conducted. The first trial was designed to evaluate the utility of 2 Fourier Transformed Infrared Microspectroscopy (FTIRM) techniques for analyzing the nutrient content of cereal grains relative to their nutritional qualities for ruminant feeding, specifically the rate and extent of rumen degradation. The second trial was designed to investigate the effects of heat processing on the ultrastructural-chemical and biological component ratio images of raw and heat-treated flax seeds using synchrotron-based FTIR microspectroscopy, including the effects of heat processing on flaxseed inherent structure of protein and CHO and on subsequent nutrient utilization and availability of flaxseed by the animal. A third study explored the chemical nature of phosphorus found in dried distiller's grains with solubles using soft X-rays of the PGM beamline to evaluate the L-edge absorption spectra for phosphorus. The objective of the study is to investigate the forms of phosphorus in wheat flour, wheat based distillers grains as well as in relevant phosphorus reference compounds in

order to understand whether the bioethanol process may increase the bioavailability of phosphorus for livestock nutrition.

The 4<sup>th</sup> study investigated the use of synchrotron radiation Fourier Transform Infrared (SR-FTIR) microspectroscopy to determine the distribution of cellulose, hemicelluloses and lignin in the cell wall of plant material. Two wheat varieties were harvested at 3 separate stages of mature (milk, early dough and maturity) and served as a source of plant material to study within and between variety differences in chemical components that make up the plant cell wall.

The results of the work detailed in the enclosed report show that:

- FTIRM analysis using both global and synchrotron-source IR light can be used to distinguish between barley varieties for important nutritional characteristics such as the endosperm starch to protein ratio which is believed to be associated with the rate of rumen degradation as measured by the *in situ* method. This would be a valuable tool for plant breeders who wish to make improvements in breeding cereal grains for feed.
- Protein secondary structure (i.e. alpha helix and or beta sheet) of heated and unheated flax seed was determined from both DRIFT and SFTIRM spectra.
- The multivariate spectral analyses of DRIFT and SFTIRM spectra of heated flaxseed showed that in terms of classification based on heat treatment and thus in terms of rumen degradability, these analyses can provide a clear idea of degradability through association to the treatment conditions.
- Phosphorus speciation in samples of grains, distillers' grains and manure from animals fed distillers' grains is clearly possible utilizing the PGM beam line at the Canadian Light Source. The phosphorus L-edge spectra indicates that there are changes in the form of phosphorus in each of these samples. However, additional data processing is necessary to understand if quantitative differences can be determined, in addition to the noted qualitative differences.

- Synchrotron FTIRM can be used to reveal spectral characteristics of aromatic lignin and cellulosic compounds and their spectral intensity ratio in the tissue cell wall of the wheat stem.
- The studies suggested that the lignin to cellulosic compounds ratio may be the best parameter to characterize the degree of lignifications of plant stems of different varieties at different growth stages and that this technique can be used to identify components of the plant associated with nutrient utilization by ruminants.

The research conducted in this study contributed to the training of two graduate students (A. Walker and K. Doiron) at the M.Sc. level and two 2 Post-doctoral students (H. Block and D. Damiran). Dr. A. Fodje was also associated with the project for a short period in 2008/09 as a research associate. To date the work has resulted in 2 peer reviewed publications (Walker et al. J. Agri & Food Chem. 2009 57:6871-6878 & Doiron et al. Spectroscopy. 2009 23:307-322) as well as 3 technical reports in the annual report of the Canadian Light Source. Further publications are expected as we prepare manuscripts on final trial work.

In summary, the results of this research shows that synchrotron-based analytical techniques can be used to understand how plant structure and composition influences nutritive value and animal performance of cattle and that this information can be used by ruminant nutritionists and plant breeders interested in developing superior feeding programs for growing and finishing cattle. Future research in this should focus on development of appropriate model for assessing nutrient utilization in the rumen that can be correlated with detailed analysis on chemical composition that is now available through appropriate beam lines of the Canadian Light Source.

## **Completed Research Trials**

### **Project 1. Use of SR-FTIR microspectroscopy as a tool for evaluating the degradation characteristics of cereal grains fed to ruminants.**

Specific objectives of this study were to evaluate the utility of FTIRM as a technique for analyzing the nutrient content of cereal grains relative to their nutritional qualities for ruminant feeding, specifically the rate and extent of rumen degradation. In order to accomplish this, it was necessary to determine the physical digestibility characteristics of cereal grains using the traditional method of analysis, the *in situ* nylon bag technique. This included a direct comparison of corn and barley, as well as a second comparison of four different varieties of barley.

This work has formed the basis of a M.Sc. project for Amanda Walker.

## **Synchrotron and Global IR Data Collection Protocol and Methodology**

### **Hypothesis**

Synchrotron radiation Fourier Transform Infrared (SR-FTIR) microspectroscopy has potential to be a tool for predicting the degradability of cereal grains in the rumen.

### **Objectives**

- To compare chemical and structural information obtained from IR analysis with physical digestibility data from nylon bag incubations.
- To determine if chemical information can be used to identify grain varieties with inherently fast or slow rates of *in situ* rumen degradation.

The research consisted of comparing two different methods of analysis, IR microspectroscopy and nylon bag digestibility, to determine if they are able to provide similar predictability regarding the digestibility characteristic of cereal grains. Infrared analysis was carried out using both a conventional global light source and synchrotron light, for the purpose of comparing their predictive capabilities.

### Seed Selection

Seed selection was biased to obtain a uniform group of kernels for analysis. Barley kernels were sieved using a Dockage Tester at the Crop Development Centre. Since there is variation in size between barley varieties, kernels from each variety were sieved on 7.5/64", 7.0/64" or 6.5/64" screens to reduce the variability in size and shape. Kernels were then weighed in order to select a sample of five seeds with the same weight and density (B. Rossnagel, personal communication).

**Table 1.** Seed populations from which kernels were selected for IR analysis.

| Variety    | Screen size (mm) | Weight (mg) |
|------------|------------------|-------------|
| Valier     | <2.77, >2.59     | 47.5–48.5   |
| Bold       | <3.18, >2.97     | 54.0–54.9   |
| Dolly      | <3.18, >2.97     | 56.0–56.9   |
| Harrington | <3.18, >2.97     | 53.0–53.9   |

### Sample Preparation for Global Analysis

Kernels of corn and barley were prepared for IR analysis by cutting thin sections to be mounted on IR microscope slides. Because the kernels are dry and desiccated, they tend to shatter when cut. To solve this problem, kernels were first be imbibed in distilled water for 24 hours at 4°C. The tips of the kernels were removed with a scalpel blade to enhance water penetration into the interior (S. Miller, personal communication). Corn kernels were rubbed with sandpaper to remove the top portion (dent area) of the structure, as cutting tends to crack the kernel. After soaking, samples were frozen at -20°C and cut into 6-10 µm sections using a cryomicrotome. Samples were mounted in OCT compound for sectioning by embedding the bottom portion of the kernel in the OCT. Sections were taken from the top portion of the kernel which is not embedded in the OCT compound, and cut approximately one-third of the distance from the top of the kernel. Figure 1 shows the approximate area from which sections were cut on a barley kernel. Corn kernels were cut in a similar manner. This area is chosen for two reasons: first, to avoid collecting cross sections that bisect the embryonic region of the kernel; and secondly to collect an area of endosperm tissue that will be representative of the maturity of the whole kernel. Starch granules at the tip of

the kernel tend to fill up last during the growth and maturation stages of the plant (B. Rossnagel, personal communication), and are therefore less dense than lower in the kernel.

Cut sections were dry mounted onto Low-e IR microscope slides for microspectroscopy in reflectance mode, or BaF<sub>2</sub> windows for transmission mode.

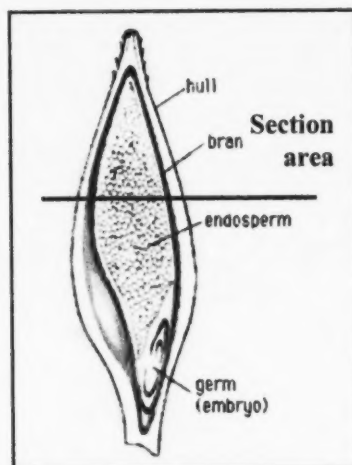


**Figure 1.** Longitudinal section of a barley kernel indicating location of cross sections to be taken for IR analysis.

### **Sample Evaluation for Globar Analysis**

The first objective of the globar analysis was to carry out a comparison of spectral quality using over-stepping technique to determine aperture and step sizes that produce optimal resolution for grain tissue. Spatial resolution is affected by the signal to noise ratio (S/N), which decreases as the aperture is closed to confine the IR beam to a smaller area. IR microscopes equipped with a conventional IR source encounter a S/N limitation at aperture settings of 20-30  $\mu\text{m}$  (Miller, 2004). With the cellular dimension of starch granules in the endosperm of cereal grain tissue being in the range of 5-10  $\mu\text{m}$ , the aperture size achievable while maintaining acceptable S/N is too large to focus on a single starch granule.

Reducing the step size between the locations of the points where spectra are collected for a map produces improved spectra by increasing the number of data points

scanned in the same area, and helps to overcome some of the inherent resolution limitations of the instrument (Mike Jackson, personal communication).

In order to determine the optimal step size that would produce the best spectral data, maps using step sizes of 50, 25 and 12  $\mu\text{m}$  were generated at an aperture setting of 50  $\mu\text{m}$ . A single section of barley tissue was used for mapping an area from pericarp to endosperm. Coordinates were saved and the same mapping area will be used for each step-size.

Using the optimal aperture and step size determined above, area maps from pericarp to endosperm from a single section of one kernel of each grain species (corn and barley) were generated for qualitative comparison of tissue components (protein, lipid, structural carbohydrate, starch, etc.). Dimensions of the mapping area were approximately 600 x 300  $\mu\text{m}$  (Figure 2).

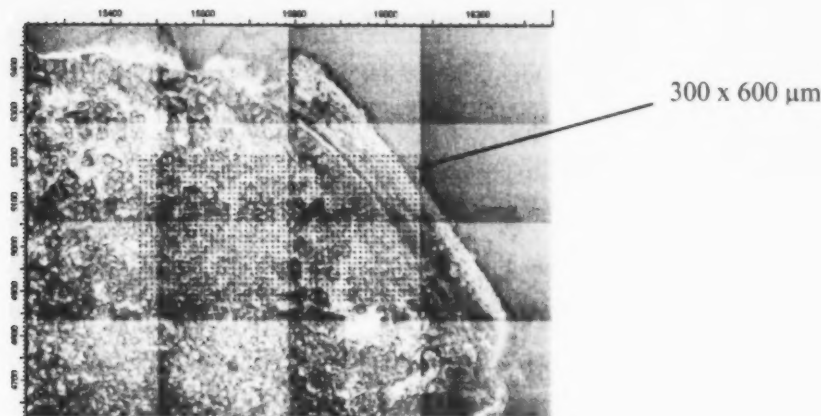


**Figure 2.** Sample visual image with scale map area.

The second objective of the global work was a quantitative comparison of starch and protein content in the endosperm tissue of corn and barley. Fifty spot samples from 5 tissue sections of the corn and barley (cv. Harrington) were collected to produce a total of 250 spectra taken from the endosperm region for each grain. All spot samples were placed between 100 and 600  $\mu\text{m}$  inside the aleurone layer in the mid-cheek region of the cross section wherever possible.

Statistical analysis was performed on this data to determine if there are significant differences in the endosperm tissue composition of different grains.

Four varieties of barley: CDC Bold, CDC Dolly, Harrington, and Valier Collect, had 50 spot samples collected to compare the differences in endosperm tissue composition between barley varieties. These varieties have been selected based on their kernel texture or hardness, as determined by the Single Kernel Characterization System (SKCS) (Osborne and Anderssen, 2003).

### **Sample Preparation for Synchrotron Evaluation**

Sections of corn, Harrington barley and Valier barley were analyzed in this experiment. From previous nylon bag studies, these three samples have been observed to exhibit the largest variation in the rate of DM degradation. Therefore, our SR-FTIR analysis was concentrated on protein and starch in these samples.

Five seeds of each grain were embedded in paraffin and cut approximately one-third of the distance from the top of the kernel in 6  $\mu\text{m}$  sections using a microtome. Figure 1 showed the approximate area from which sections were cut on a barley kernel. Corn kernels were cut in a similar manner.

One section from each seed was fixed onto a BaF<sub>2</sub> transmission window for analysis in transmission mode. Two or three sections from the same seeds were also fixed onto LowE-IR slides for analysis in reflectance mode as an alternative to the transmission slides in the case of instrumentation problems at the beamline.

### **Data Collection**

#### **Spot Samples**

Spot samples were collected from sections of corn, Harrington barley and Valier barley. Thirty spot samples from each of 5 tissue sections of three grains (corn, Harrington, Valier) were scanned, for a total of 450 spot samples (150 from each of the three grains) (Figure 3). Synchrotron spectra were intended to be collected from the same location as global spectra, however instrument limitations prevented this.

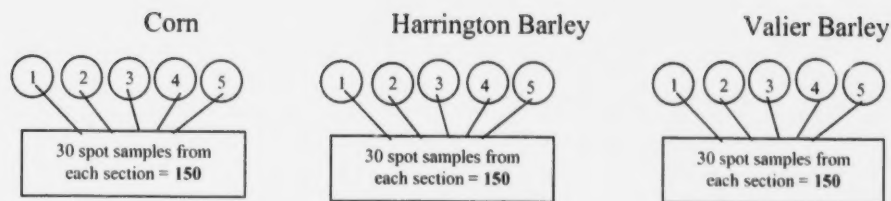


**Figure 2.** Sample disks for transmission scanning.

#### Instrument Optimization

- Beam signal amplitude was maximized at each point by placing the each analysis point individually and re-focusing the microscope on each point.
- A new background spectrum was collected after every 10 to 15 spot samples.
- The optimum sample scan number (ie. 32 vs. 64 vs. 256) was determined experimentally in order to optimize spectral quality.
- A 10 x 10  $\mu\text{m}$  aperture setting was to be used as it is within the cellular dimensions of the tissue being studied and maintains an acceptable S/N ratio (Yu et al, 2004a). Spectra were collected in the mid-infrared range 4000 to 800  $\text{cm}^{-1}$  at a resolution of 4 $\text{cm}^{-1}$ .

#### Mapping Data

One large map from one section of each grain was collected from the outer seed coat into the interior endosperm area to observe the differences in chemical composition between tissues. Approximate map dimensions will be 300 x 600  $\mu\text{m}$ , or larger depending on the time required to collect the data (Figure 2). If time permitted, smaller area maps (approx. 50 x 50  $\mu\text{m}$ ) specific areas of interest were to be collected. Examples would include interior endosperm, sub-aleurone endosperm, aleurone layer, pericarp and seed coat (Figure 3). Synchrotron based maps will be qualitatively compared to global based maps.

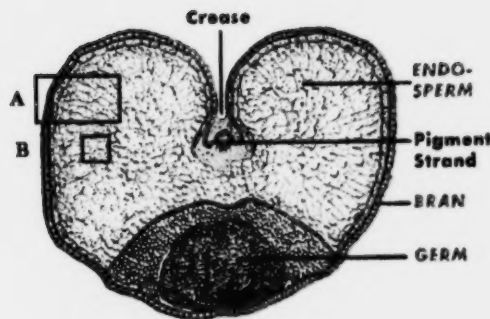


**Figure 3.** Cross section of wheat kernel indicating where area maps were taken. A) map from pericarp into endosperm and B) map of endosperm only (Maps are not to scale).

### **Nylon Bag Digestibility Trial**

Rumen degradation rates were measured and comparisons made between barley and corn and between different barley varieties. Both ground and rolled samples were evaluated to ascertain the comparative accuracy of digestibility estimates obtained with *in situ* methods. First, a trial was conducted to compare the degradability of corn (cv. Pioneer 39P78) and barley (cv. Dolly). A second trial looked at the differences in degradability of four different barley cultivars that have been rated on the basis of kernel texture: CDC Bold (softest), Harrington, Valier, and CDC Dolly (hardest). Dolly was included in both trials as a means of comparison between the results obtained in each trial.

### **Sample Preparation**

Dry-rolling and grinding were chosen as processing methods for this experiment. These were chosen to alter particle size and access of rumen bacteria to the starch and protein matrix of the endosperm such. Ground barley and corn samples were processed through a 2 mm screen as reported by Mustafa et al. (2000) and Boss and Bowman (1996). For the rolled treatments, samples of corn (cv. Pioneer 39P78) and barley (cv. Harrington, Dolly, Valier, and Bold) were coarsely processed with a roller mill, and a representative sample collected for *in situ* incubation. Prior to rolling, dry

barley samples were tempered to approximately 10-12% moisture according to the AACC method 26-95 (Test Milling Tempering Table).

In order to obtain the most uniform sample possible, seed samples were screened to remove small and large seeds that would be differentially affected by processing. As per the IR analysis portion, seed samples were screened and the portion of kernels within the size ranges detailed in Table 1 were used for the *in situ* trials.

Rumen degradation characteristics were determined using the nylon bag *in situ* technique. Ten treatments include rolled and ground samples of one variety of corn (cv. 39P78) and four varieties of barley (Harrington, Valier, Dolly, and Bold).

#### *Animals*

Two Angus yearling heifers fitted with rumen fistulas were used for the *in situ* trials. Heifers were fed a typical feedlot finishing diet consisting of 85% concentrate and 15% barley silage at 2.2% of body weight. The concentrate was composed of equal amounts of rolled corn and rolled barley.

#### *Rumen Incubation*

Ruminal degradation characteristics were determined using the nylon bag method as described by Yu et al. (2003). Two separate incubation trials were conducted: one comparing ground and rolled corn and Harrington barley, and the second comparing four varieties of ground and rolled barley (Harrington, Valier, Dolly and Bold).

After incubation, all bags were removed from the rumen and rinsed in cold water to stop microbial activity and remove excess ruminal contents. 'Check' bags (those incubated for 0 hours) were included to determine the washout rate of material from the bags to allow a correction factor to be applied to other treatments. All bags were oven dried in at 55 °C for 48 hours, and then frozen until analysis. In order to obtain sufficient sample size for chemical analysis, dry residues were pooled according to grain variety, incubation time and animal.

### *Chemical Analysis*

Samples for laboratory analysis were prepared by grinding through a 1mm screen. Dry matter and crude protein will be analyzed according to procedures of the AOAC (1990). Starch content will be analyzed using the Megazyme Total Starch Assay Kit.

### **Results and Discussion**

#### **In Situ Incubations of Cereal Grain Samples:**

Grinding causes a reduction in particle size that tends to increase the rate and extent of digestion beyond that which occurs when feed is masticated and ruminated by the animal. Such overprocessing of grain in commercial situations can lead to a reduction in feed value by decreasing feed intake and causing digestive upsets (Wang and McAllister, 2000); therefore, grinding is not used as a common processing method for ruminant feeding. However, the inclusion of ground samples in digestibility experiments can be of value when assessing the rates of *in situ* dry matter, protein and starch degradability of cereal grains. Processing effects on the structure and particle size of cereal grains relative to the rate of *in situ* degradation have been established by previous work. The objective of this experiment was not to compare the differences in rate of degradation among processing methods as this has been studied by previous researchers (Theurer, 1986; Wang and McAllister, 2000; Ramsey et al., 2001); rather, the intent was to examine the effect of endosperm structure on the rate and extent of digestion of cereal grains and if the processing affected different grain varieties in the same manner. Since the premise of this study was to determine if the inherent chemical structure of the endosperm tissue has an effect on the rate of degradation, it was hypothesized that grinding should destroy most of the protein-starch structures that would differentiate the rate of digestion of one barley variety from another. Therefore, with ground samples it was anticipated that all varieties would show more similar rates of degradation. Differences were expected to be observed among the rolled treatments as this processing method does not destroy the inherent structures of the endosperm.

Ground barley and corn samples were processed through a 2mm screen as reported by Mustafa et al. (2000) and Boss and Bowman (1996). Other researchers

have used a 3mm grind for *in situ* samples (Lehman et al., 1995; Ramsey et al., 2001), but did not report any significant differences in digestibility due solely to the particle size. Ramsey et al. (2001) found that the sample washout loss with a 3 mm grind did not differ from results obtained in previous studies carried out by the same group with 2 mm ground samples.

Particle size is not the only source of variation encountered with this experimental method. Other factors to consider when comparing degradability results from different studies include bag and pore size, sample: bag area ratio, researcher's technique and individual animal differences. In this experiment, the sample: bag surface area ratio was 0.0175 g/cm<sup>2</sup> with a pore size of 41 µm which is similar to that reported by other researchers. For example, the following sample:bag surface area ratios and bag pore sizes, respectively, have been reported: 0.0125 g/cm<sup>2</sup> and 51 µm, 0.050 g/cm<sup>2</sup> and 57 µm, 0.0286 g/cm<sup>2</sup> and 57.5 µm, and 0.026 g/cm<sup>2</sup> and 50 µm (Lehman et al., 1995; Yang et al., Khorasani et al., 2000; 1996; and Ramsey et al., 2001, respectively).

### **Trial 1: Comparison of Corn and Harrington Barley**

Starch and crude protein content (DM basis) as well as the ratio of starch:protein content in the entire kernel for corn and the four barley cultivars used for the *in situ* experiment are shown in Table 2.1. Harrington and Valier barley had similar chemical composition with regard to starch and crude protein content, analogous to the findings of Yu et al. (2003). Starch content of Harrington and Valier was also similar to the average of 60 barley varieties reported by Khorasani et al. (2000). CDC Bold had the highest starch content of the four barley varieties (63%), but the lowest CP (11%), which resulted in the highest starch:protein ratio. CDC Dolly was intermediate between CDC Bold and Harrington and Valier for both starch and protein content (58.7% and 12.8%, respectively). Corn had higher starch content than the four barley varieties, and lower protein than all barley varieties except CDC Bold.

Protein within the endosperm tissue of cereal grains is arranged in a matrix that surrounds and protects the starch granules, making them less available for digestion by rumen bacteria (McAllister et al., 1993). This may be a major factor responsible for differences in rumen starch degradation among different grain varieties. If more protein within the grain kernel relates to a stronger protein matrix, higher protein content should indicate a larger degree of 'protection' which would be related to a reduction in the rate and extent to which starch is able to be degraded. Therefore, a lower starch to protein ratio may be indicative of closer association of starch granules with the protein matrix, resulting in a lower rate and extent of rumen degradation (Yu et al., 2004). 'Wet' chemical analysis requires homogenization of the sample tissue before analysis, so the starch:protein ratio reported in Table 2.1 reflects the relative amounts within the entire kernel, not a specific region of tissue (ie. the endosperm).

*In situ* rumen kinetics of DM, CP and starch for rolled and ground samples of corn and Harrington barley are shown in Table 2.2. The variables  $K_d$ , S and D represent an attempt to describe rumen disappearance of DM, CP and starch over a 24h period as a result of bacterial fermentation activity. The S variable represents the soluble, or 'wash-out' fraction (g/kg) of the feed (measured for each of DM, CP and starch), and is defined as 100 minus the residue remaining in the 0h bags after the washing procedure (i.e. the soluble portion of sample washed out of the bags without being subjected to rumen incubation). Feed material which is potentially rumen degradable corresponds to the D (g/kg) variable, while the  $K_d$  represents the rate, expressed as  $\% h^{-1}$ , at which material (DM, CP or starch) is degraded in the rumen environment. These variables are then used to predict the effective degradability of DM, CP and starch in the rumen assuming a fixed rumen passage rate of  $6\% h^{-1}$  (Yu et al., 2003). From the point of view of this work, the rate and extent of rumen degradation (ie. effective degradability) are the two most critical factors.

As expected, the rate of DM degradation ( $K_d$ ) was significantly lower ( $P < 0.05$ ) for corn than barley (Herrera-Saldana et al., 1990; Boss and Bowman, 1996), and lower for rolled than ground samples. There was, however, a significant interaction of the effects of grain type and processing method ( $P < 0.05$ ) on rate of rumen DM degradation. Both GC and GH showed faster rates of degradation than RC and RH

(9.6 and 31.3 vs 8.9 and 12.1 % h<sup>-1</sup>, respectively), but the magnitude of the processing effect was different for each grain. Grinding significantly increased the K<sub>d</sub> of DM for barley from 12.1 % h<sup>-1</sup> for RH to 31.3 % h<sup>-1</sup> for GH (P<0.05), while for corn, there was no statistical difference between RC (8.9 % h<sup>-1</sup>) and GC (9.6 % h<sup>-1</sup>). A similar interaction between grain type and processing method was seen in the ruminal degradation rate (K<sub>d</sub>) of both crude protein and starch as was seen for DM. The nature of these interactions is illustrated in Figure 2.1. In general, the effect of processing on the rate of DM, CP and starch degradation was a greater for Harrington than corn.

Intuitively, ground corn should be more quickly degraded and to a greater extent than rolled corn, as seen for barley, but the lack of difference in their rate of degradation is not necessarily surprising. Dry-rolled and ground corn are known to have a lower rate of digestibility than corn processed with heat and steam (Theurer, 1986). A 9-year summary of published trials with corn grain by Huntington (1995) reported that steam flaking increased starch digestibility an average of 7% over dry processing. As opposed to barley, reduction in particle size does not have as much effect on the starch degradability of corn as does the application of moisture, heat and pressure during processing (Yu et al., 1998).

There was a significant grain type \* processing interaction observed for EDDM (Figure 2.1). The nature of this interaction was such that the EDDM of ground Harrington increased over rolled Harrington to a greater degree than seen with corn. A significant interaction was not detected for EDCP or EDSt. For both parameters, a greater (P<0.05) response was observed with fine processing and for Harrington relative to corn (Figure 2.1). These results indicate that measuring DM degradation kinetics may be indicative of the rate and extent of starch digestion. However, this is opposite to the observations of Ramsey et al. (2001), who found no correlation between the rate of DM and starch disappearance in either ground or rolled grain. These researchers concluded that measurement of DM disappearance was not adequate for estimating ruminal starch disappearance in barley grain.

**Table 2.1.** Nutrient composition of corn and barley samples used for experimental work.

| Item                        | Grain |          |           |            |        |
|-----------------------------|-------|----------|-----------|------------|--------|
|                             | Corn  | CDC Bold | CDC Dolly | Harrington | Valier |
| DM (%) <sup>y,z</sup>       | 90.59 | 88.27    | 88.39     | 88.92      | 89.20  |
| Starch (% DM) <sup>z</sup>  | 66.52 | 63.32    | 58.74     | 57.78      | 56.61  |
| CP (% CM) <sup>z</sup>      | 11.49 | 11.10    | 12.78     | 14.06      | 14.01  |
| Starch:Protein <sup>x</sup> | 5.79  | 5.70     | 4.59      | 4.11       | 4.04   |

<sup>z</sup> Samples run in duplicate, accepted at an error rate of <3%.

<sup>y</sup> Moisture content (%) of grain after processing and prior to incubation

<sup>x</sup> Ratio refers to starch and protein content of entire kernel

**Table 2.2.** Trial 1 *in situ* rumen degradation kinetics for corn and Harrington barley.

| Item                                               | Corn    |         | Harrington |         | SEM   | P-value |            |        |
|----------------------------------------------------|---------|---------|------------|---------|-------|---------|------------|--------|
|                                                    | Rolled  | Ground  | Rolled     | Ground  |       | grain   | processing | g x p  |
| Rumen degradation characteristics of dry matter    |         |         |            |         |       |         |            |        |
| K <sub>d</sub> (%/h)                               | 8.85 c  | 9.64 c  | 12.11 b    | 31.29 a | 1.225 | <.0001  | <.0001     | <.0001 |
| S (%)                                              | 8.91 c  | 19.9 b  | 8.96 c     | 29.61 a | 0.474 | <.0001  | <.0001     | <.0001 |
| D (%)                                              | 50.50 c | 61.31 b | 65.90 a    | 57.46 b | 1.616 | 0.0012  | 0.3644     | <.0001 |
| EDDM (%)                                           | 38.18 d | 57.44 b | 52.65 c    | 77.79 a | 0.715 | <.0001  | <.0001     | 0.0002 |
| Rumen degradation characteristics of crude protein |         |         |            |         |       |         |            |        |
| K <sub>d</sub> (%/h)                               | 4.94 b  | 5.39 b  | 7.32 b     | 14.19 a | 0.692 | <.0001  | 0.0003     | 0.0008 |
| S (%)                                              | 8.77    | 18.14   | 22.36      | 20.42   | 3.945 | 0.0728  | 0.3663     | 0.1815 |
| D (%)                                              | 63.06   | 75.89   | 62.94      | 77.26   | 7.232 | 0.6707  | 0.2478     | 0.4544 |
| EDCP (%)                                           | 35.03   | 54.06   | 55.99      | 74.63   | 2.572 | <.0001  | <.0001     | 0.9394 |
| Rumen degradation characteristics of starch        |         |         |            |         |       |         |            |        |
| K <sub>d</sub> (%/h)                               | 6.78 b  | 8.87 b  | 12.87 b    | 39.58 a | 1.028 | <.0001  | <.0001     | <.0001 |
| S (%)                                              | 5.57    | 16.41   | 24.46      | 33.23   | 1.272 | <.0001  | <.0001     | 0.4206 |
| D (%)                                              | 68.91   | 75.92   | 64.58      | 66.2    | 4.776 | 0.0988  | 0.2877     | 0.4976 |
| EDSt (%)                                           | 39.72   | 61.29   | 68.23      | 90.69   | 0.911 | <.0001  | <.0001     | 0.5700 |

Means with different letters in the same row are significantly different (P<0.05)

K<sub>d</sub> = rate of rumen degradation of the D fraction; S = soluble or 'wash out' fraction; D = insoluble but potentially degradable fraction;

EDDM = effective degradability of DM

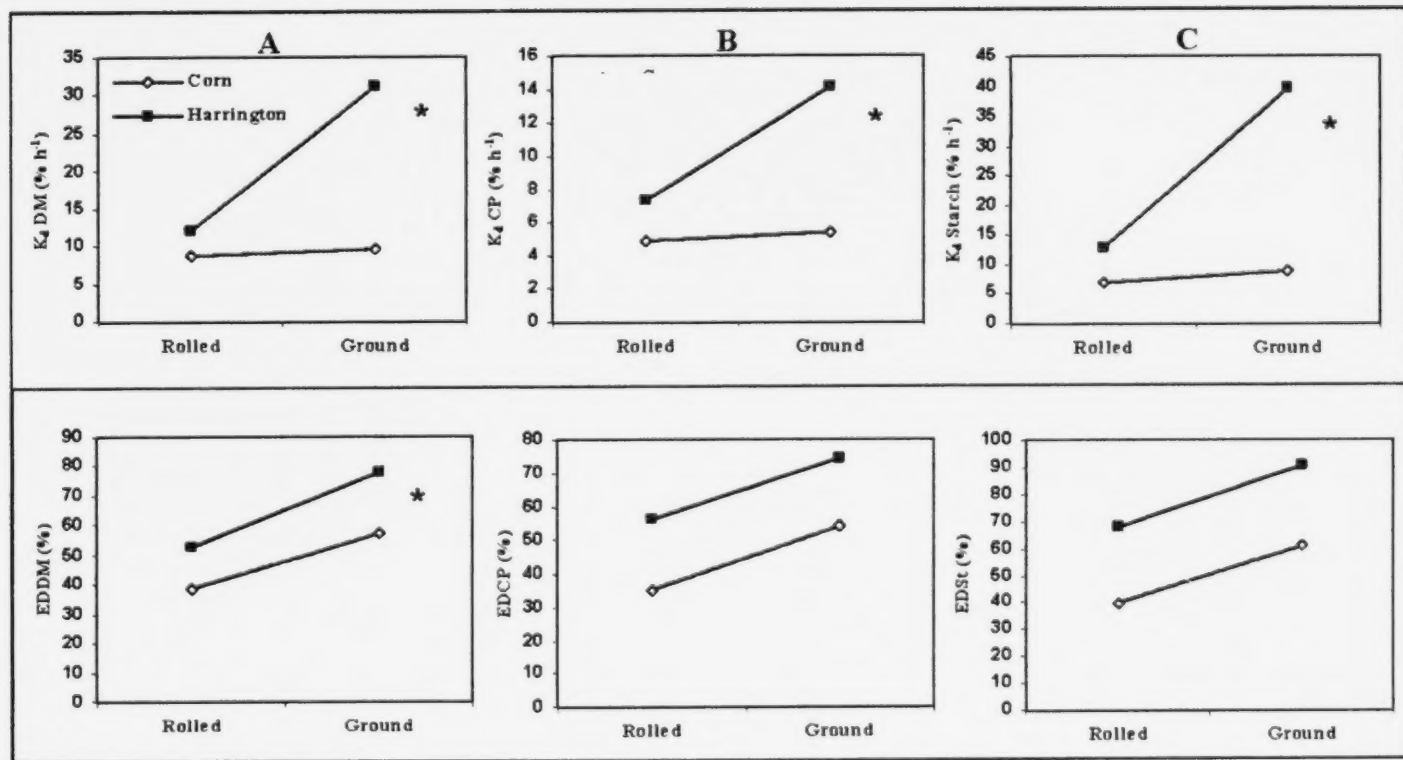


**Figure 2.1.** Comparison of the rate of degradation ( $K_d$ ) and effective degradability (ED) of A) dry matter, B) crude protein (CP); and C) starch. \* Significant interaction ( $P < 0.05$ ) between the effects of grain type and processing method.

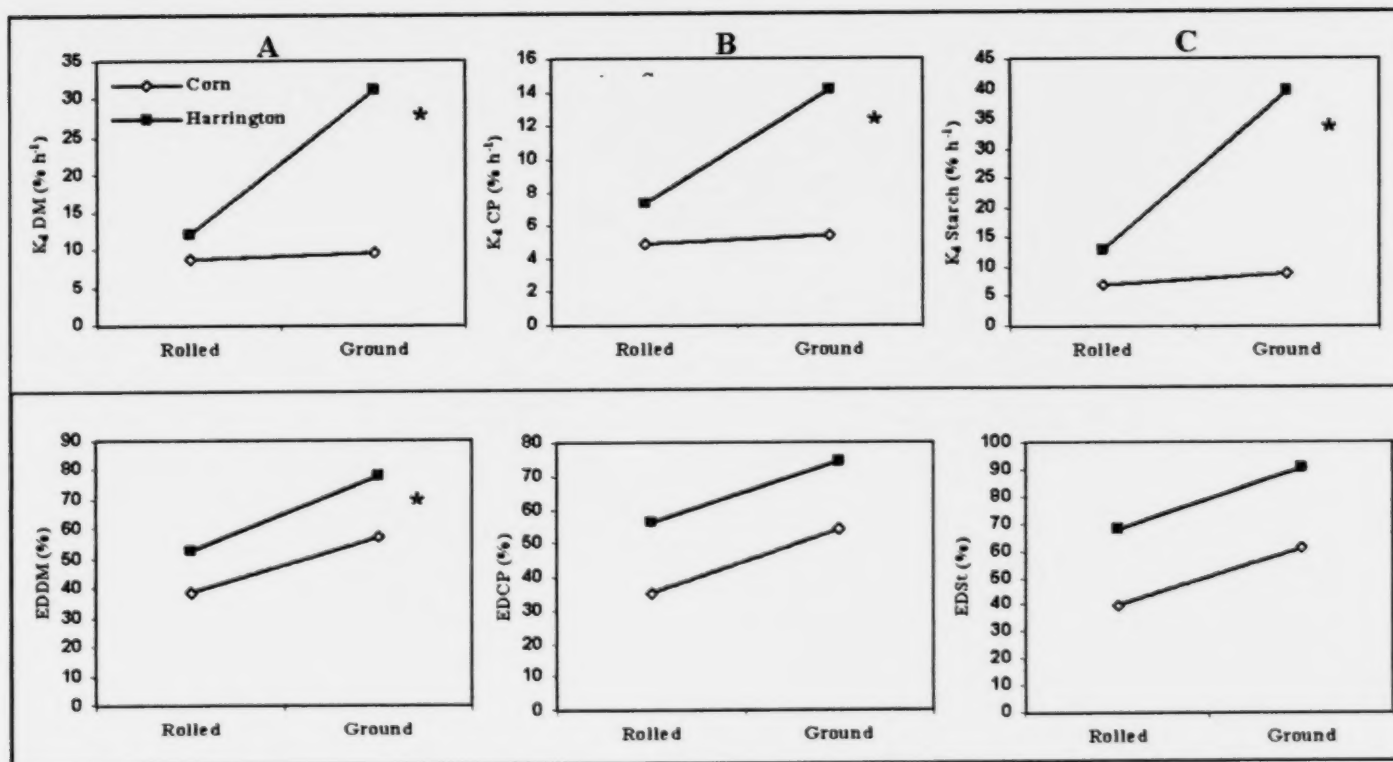


**Figure 2.1.** Comparison of the rate of degradation ( $K_d$ ) and effective degradability (ED) of A) dry matter, B) crude protein (CP); and C) starch. \* Significant interaction ( $P < 0.05$ ) between the effects of grain type and processing method.

Solubility of DM increased with reduced particle size in the ground samples compared to the rolled treatments. The soluble fraction (S) of DM was not different between RC and RH (8.91 vs 8.96%), but ground samples had a significantly higher S fraction than the rolled, and ground barley was significantly higher than ground corn ( $P < 0.05$ ). With this technique, the soluble fraction of the grain is estimated by measuring the amount of the sample that is 'washed out' of the non-incubated (0h) nylon bags during the washing procedure. Grain particle size as well as pore size of the bag are two factors that will influence this value. In this experiment, the measured pore size of the bag material was 41  $\mu\text{m}$ , and any sample particles smaller than this would potentially be washed out of the bags. Since ground barley showed significantly higher 'wash-out' fraction than ground corn, it would indicate that barley has a greater tendency to shatter and that grinding produced more excessively small particles (ie. less than 41  $\mu\text{m}$ ) than with corn. Difference in oil content between corn and barley may affect the observed response to grinding. Although crude fat analysis was not performed on these particular samples, in general corn grain contains approximately twice as much oil as does barley (4.0 vs 2.2%, respectively) (NRC, 1996).

As previously stated, the objective for including rolled and ground treatments in this experiment was to determine the processing effect on the rate and extent of rumen degradation, and whether minimal (rolling) or excessive (grinding) processing has a differential effect on the inherent degradability characteristics of cereal grains in the rumen. The results indicated a significant ( $P < 0.05$ ) grain type by processing interaction on the rate of DM, CP and starch degradation indicating that corn and barley do respond differentially to processing. However, it is important to note that the interaction was related to the magnitude of the response, but not the direction of the response. That is to say, grinding caused an increase in the rate and extent of rumen degradation in both corn and barley, but the magnitude of the response was different for each grain type. Therefore, since a similar response was observed for both processing methods, we elected to focus on the main effect of grain type by removing the ground treatment. In commercial situations, ground grain is not fed to ruminants, so from a practical point of view we wanted to focus more specifically on rolled grain as it is utilized in ruminant rations.

Table 2.3 compares the rumen degradation kinetics of rolled corn and Harrington barley. Overall, the main observations were that 1) DM, CP and starch in barley was degraded more quickly ( $P < 0.05$ ) than corn, and 2) the extent of degradation (effective degradability) was higher for barley than corn, both of which were similar to previous *in situ* digestibility experiments using ground samples (Herrera-Saldana et al., 1990; Boss and Bowman). Solubility of DM was not different between Harrington and corn. The soluble fraction of starch was significantly lower for rolled corn than Harrington (5.57 vs 24.5%), and the solubility of CP followed a similar trend, although not significant ( $P = 0.115$ ). Previous reports have indicated that the protein matrix must first be disrupted before starch granules can be released and made available for digestion (McAllister and Cheng, 1996), therefore, a reduction in protein solubility may cause a subsequent reduction in the degradation of starch. Effective degradability of CP and starch was lower for corn (35.0 and 39.8%) than Harrington barley (56.0 and 68.2%), as a result of slower rate of degradation, leaving more rumen undegradable material and reducing the overall degradability. Effective rumen degradability of starch (EDSt) was nearly 70% greater ( $P < 0.05$ ) for RH than RC (Table 2.3). This agrees with Ørskov's (1986) observation that a greater proportion of barley starch is fermented in the rumen than that of corn. Despite corn having a higher overall starch content than Harrington barley (66.5 vs 57.8%, respectively) (Table 2.1), a larger fraction of corn starch escapes ruminal fermentation. McAllister et al. (1993) reported that bacterial attachment to and digestion of starch granules in the rumen are hindered by the surrounding protein matrix, and that this interaction has a larger effect on the digestion of starch in corn than in barley. This could be a causative factor in the differences seen between the rumen degradation rate of corn and Harrington barley in this experiment.

**Table 2.3.** Trial 1 *in situ* rumen degradation kinetics for rolled samples of corn and Harrington barley.

| Item                                                      | Grain type |            | SEM    | P-value |
|-----------------------------------------------------------|------------|------------|--------|---------|
|                                                           | Corn       | Harrington |        |         |
| <i>Rumen degradation characteristics of dry matter</i>    |            |            |        |         |
| K <sub>d</sub> (%/h)                                      | 8.85 a     | 12.11 b    | 1.330  | 0.0046  |
| S (%)                                                     | 8.91       | 8.96       | 0.401  | 0.9276  |
| D (%)                                                     | 50.50 b    | 65.90 a    | 2.007  | 0.0033  |
| EDDM (%)                                                  | 38.18 b    | 52.65 a    | 0.807  | 0.0001  |
| <i>Rumen degradation characteristics of crude protein</i> |            |            |        |         |
| K <sub>d</sub> (%/h)                                      | 4.94 b     | 7.32 a     | 0.8711 | 0.0411  |
| S (%)                                                     | 8.77       | 22.36      | 5.215  | 0.1149  |
| D (%)                                                     | 63.06      | 62.94      | 9.842  | 0.9916  |
| EDCP (%)                                                  | 35.03 b    | 55.99 a    | 3.504  | 0.0055  |
| <i>Rumen degradation characteristics of starch</i>        |            |            |        |         |
| K <sub>d</sub> (%/h)                                      | 6.80 b     | 12.87 a    | 1.173  | 0.0039  |
| S (%)                                                     | 5.57 b     | 24.46 a    | 1.077  | 0.0006  |
| D (%)                                                     | 68.91      | 64.59      | 5.774  | 0.5812  |
| EDSt (%)                                                  | 39.82 b    | 68.23 a    | 1.098  | 0.0002  |

Means with different letters in the same row are significantly different (P<0.05)

## **Trial 2: Comparison of four barley varieties**

Effects of barley variety and processing method (rolled vs ground) on the *in situ* DM degradation characteristics of DM, CP and starch are presented in Table 2.4. Again, the rationale for including both processing methods was to determine if these different barley varieties would exhibit different responses to digestion in the rumen at different levels of processing. Table 2.4 shows that there were variety and processing effects as well as variety by processing interactions for nearly all parameters measured. Fine processing (grinding) doubled the rate of digestion of DM from 15.3% h<sup>-1</sup> for rolled samples to 33.3% h<sup>-1</sup> for ground samples (average K<sub>d</sub> of four varieties), and increased the extent of DM degradation by approximately 20% (average EDDM = 78.2 vs 59.4% for grinding and rolling, respectively). Variety differences were primarily seen between two groups: CDC Bold and CDC Dolly differing from Harrington and Valier. However, of most importance is the interaction between barley variety and processing methods observed for both rate and extent of DM, CP and starch degradation. Processing produced a similar directional response in the K<sub>d</sub> and effective degradability of DM, CP and starch for all barley varieties, whereas the magnitude of the response varied between varieties. These interactions are illustrated in Figure 2.2. Grinding caused the rate of degradation of CP to double for CDC Bold and CDC Dolly, but did not produce any significant increase in the K<sub>d</sub> of CP over the rolled treatment for Harrington or Valier (Table 2.4). A wide variation existed in the rate of CP digestion in ground samples, ranging from 30.5% h<sup>-1</sup> for CDC Bold, to 12.4% h<sup>-1</sup> for Harrington (Table 2.4). Ground Harrington and Valier were statistically the same for rate of CP degradation, and significantly lower than ground samples of both CDC Dolly and CDC Bold. The effect of grinding on the rate CP degradation may have implications with respect to the rate of starch digestion. As noted by McAllister et al. (1995), the protein matrix appears to serve as a protective barrier to starch digestion, therefore as the rate of disappearance of CP increases, the rate of starch disappearance should follow the same pattern. This trend appears to occur in this experiment as the rate of starch degradation for CDC Bold and CDC Dolly is higher (P<0.05) than that of Harrington. The K<sub>d</sub> of

**Table 2.4.** *In situ* rumen degradation characteristics of DM, CP and starch of ground and rolled samples from four barley varieties.

| Item                                                      | Barley variety |         |          |          |            |         |          |         | SEM   | P-value |        |        |
|-----------------------------------------------------------|----------------|---------|----------|----------|------------|---------|----------|---------|-------|---------|--------|--------|
|                                                           | Bold           |         | Dolly    |          | Harrington |         | Valier   |         |       | v       | p      | v x p  |
|                                                           | Rolled         | Ground  | Rolled   | Ground   | Rolled     | Ground  | Rolled   | Ground  |       |         |        |        |
| <i>Rumen degradation characteristics of dry matter</i>    |                |         |          |          |            |         |          |         |       |         |        |        |
| K <sub>d</sub> (%/h)                                      | 16.61 c        | 38.35 a | 15.77 c  | 36.95 a  | 14.92 c    | 28.40 b | 13.71 c  | 29.35 b | 1.534 | 0.0006  | <.0001 | 0.0295 |
| S (%)                                                     | 10.50 d        | 37.22 a | 9.25 d   | 30.23 b  | 9.42 d     | 29.28 b | 6.68 e   | 27.71 c | 0.815 | <.0001  | <.0001 | <.0001 |
| D (%)                                                     | 70.48 a        | 49.31 c | 71.75 a  | 57.73 b  | 70.23 a    | 57.26 b | 69.67 a  | 59.08 b | 1.179 | 0.0006  | <.0001 | 0.0004 |
| EDDM (%)                                                  | 62.2 c         | 79.82 a | 61.01 cd | 79.88 a  | 59.30 d    | 76.53 b | 55.01 e  | 76.51 b | 0.950 | <.0001  | <.0001 | 0.0313 |
| <i>Rumen degradation characteristics of crude protein</i> |                |         |          |          |            |         |          |         |       |         |        |        |
| K <sub>d</sub> (%/h)                                      | 12.24 c        | 30.50 a | 11.77 c  | 25.57 b  | 10.11 c    | 12.38 c | 10.15 c  | 15.71 c | 1.589 | <.0001  | <.0001 | <.0001 |
| S (%)                                                     | 7.04 d         | 27.32 a | 4.71 de  | 21.19 b  | 2.74 ef    | 23.35 b | 0.56 f   | 16.41 c | 1.353 | <.0001  | <.0001 | 0.0354 |
| D (%)                                                     | 77.79 ab       | 60.78 d | 79.64 a  | 72.67 bc | 78.73 a    | 71.82 c | 78.09 ab | 78.92 a | 2.00  | 0.0009  | <.0001 | 0.0019 |
| EDCP (%)                                                  | 59.16 d        | 77.75 b | 57.19 d  | 79.92 a  | 52.01 e    | 71.55 c | 48.49 f  | 72.97 c | 1.207 | <.0001  | <.0001 | 0.0224 |
| <i>Rumen degradation characteristics of starch</i>        |                |         |          |          |            |         |          |         |       |         |        |        |
| K <sub>d</sub> (%/h)                                      | 16.98 bc       | 46.32 a | 16.56 bc | 46.24 a  | 16.67 bc   | 29.69 b | 14.75 c  | 37.80 a | 3.601 | 0.0317  | <.0001 | 0.0393 |
| S (%)                                                     | 11.39 e        | 44.04 a | 8.40 f   | 35.89 b  | 9.56 ef    | 33.09 c | 4.01 g   | 28.02 d | 1.148 | <.0001  | <.0001 | 0.0002 |
| D (%)                                                     | 81.85 a        | 55.32 e | 85.15 a  | 63.62 d  | 81.01 b    | 65.96 d | 85.25 a  | 71.28 c | 1.315 | <.0001  | <.0001 | <.0001 |
| EDSt (%)                                                  | 71.86          | 92.95   | 70.75    | 92.18    | 68.94      | 76.80   | 64.44    | 89.41   | 4.459 | 0.1483  | <.0001 | 0.2606 |

Means with different letters in the same row are significantly different (P&lt;0.05)

v - the effect of variety; p - the effect of processing

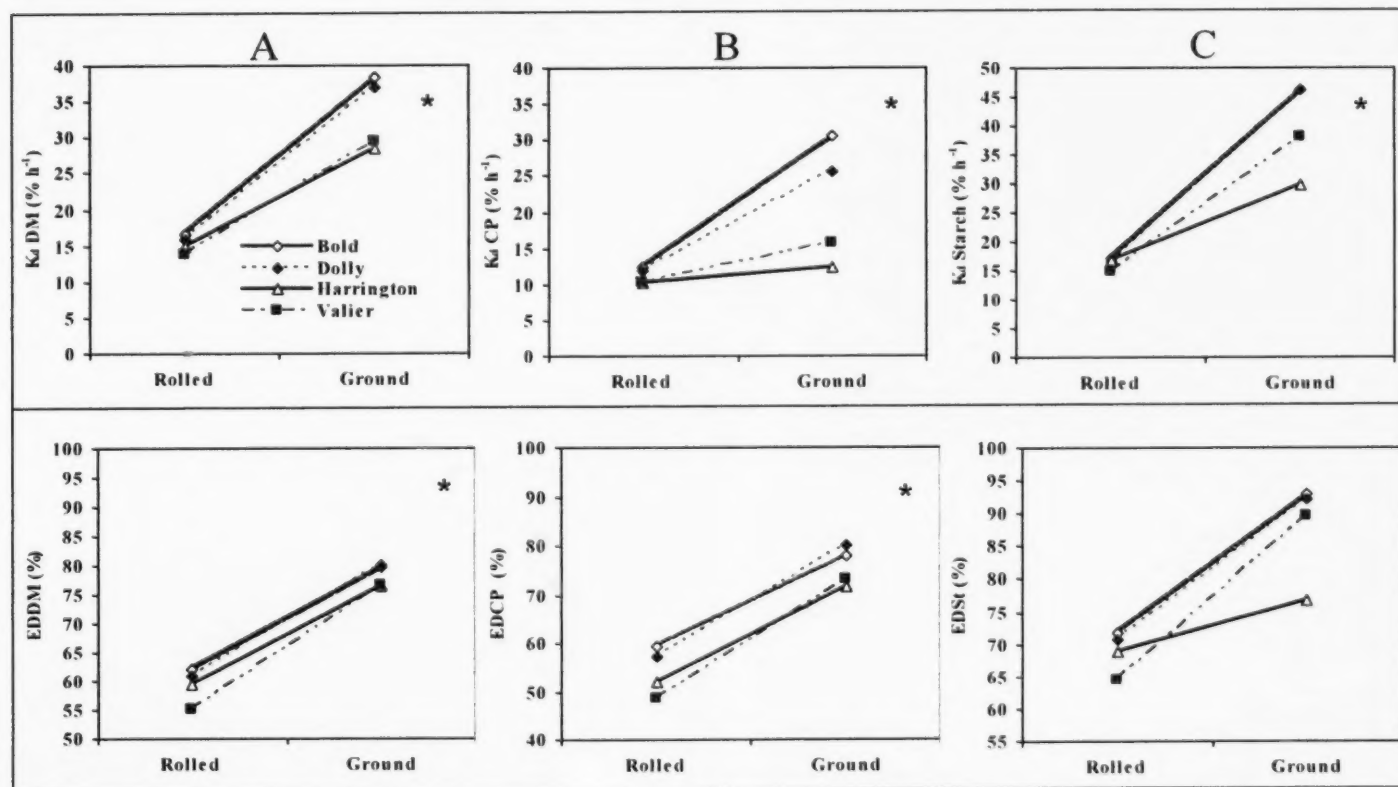


**Figure 2.2.** Comparison of the *in situ* rate of degradation ( $K_d$ ) and effective degradability (ED) of A) dry matter, B) crude protein (CP); and C) starch of four barley varieties: CDC Bold, CDC Dolly, Harrington and Valier. \* Indicates a significant interaction ( $P < 0.05$ ) between the effects of grain type and processing method.

starch for ground Valier is numerically lower than that of CDC Bold and CDC Dolly, but is not statistically different.

Grinding also increased the EDCP of Valier to a greater extent (>11%) than seen with the other barley varieties. In total, the EDCP of ground Valier increased by nearly 50% over the rolled sample, while CDC Bold, CDC Dolly and Harrington were increased by 30, 37 and 39%, respectively. Processing also increased the rate of starch degradation, but once again, the magnitude of the effect was not equal for all varieties. CDC Bold and CDC Dolly showed an increase in the  $K_d$  of starch by a factor of 1.7 and Valier increased by a factor of 1.5, while the starch  $K_d$  for ground Harrington was only increased by a factor of 0.8 over the rolled treatment.

Rates of DM disappearance from ground barley samples ranged from 28.4 to 38.5%  $h^{-1}$ , similar to rates reported by Lehman et al. (1995; 23 to 35%  $h^{-1}$ ), Yu et al. (2003; 29 to 32%  $h^{-1}$ ) and Khorasani et al. (2000; 38%  $h^{-1}$ ). Ramsey et al. (2001) observed much higher rates of DM degradation ranging from 33 to 58%  $h^{-1}$ . It seems that all these studies show a high degree of variability in the measurements, increasing the range in digestibility rate and making it more difficult to find statistical differences.

In contrast to the results of this experiment, neither Ramsey et al. (2001), Boss and Bowman (1996) nor Yu et al. (2003) were able to detect an effect of barley variety on the rate of DM disappearance of ground barley grains. Lehman et al. (1995), did observe an effect of barley variety on the rate of DM disappearance, however, their study compared 22 different varieties of barley, thus increasing the probability of detecting statistical differences.

The expectation in this experiment was that grinding would tend to reduce the variability in rate and extent of rumen degradation among barley samples by destroying the physical structure of the endosperm tissue, thus making the starch more uniformly available to rumen bacteria for the process of fermentation. However, we observed statistical differences among ground samples that did not occur among the rolled samples. Ground samples of CDC Bold and CDC Dolly showed higher ( $P<0.05$ ) rates of rumen disappearance than Harrington and Valier for both DM (38.3 and 36.9 %  $h^{-1}$  vs. 28.40 and 29.35 %  $h^{-1}$ , respectively) and CP degradation (30.5 and 25.6 %  $h^{-1}$  vs.

28.4 and 29.4 % h<sup>-1</sup>, respectively) (Table 2.4). This result was consistent with the degradation rates of 32 % h<sup>-1</sup> for Harrington and 29 % h<sup>-1</sup> for Valier (Yu et al. 2003).

Solubility (S) of DM from ground barley ranged from 28.4 to 36.9%, with a mean value of 31% (Table 2.4.); similar to the 33.7% soluble fraction of DM measured by Lehman et al. (1995), and slightly lower than 35.5% soluble fraction obtained by Yu et al. (2003). Others have reported a noticeably higher proportion of the 'wash-out', or soluble DM fraction. Ramsey et al. (1995) measured an average of 51% in the wash-out fraction of ten barley varieties, while in a comparison of sixty barley cultivars by Khorasani et al. (2000), the DM solubility was found to range from 35.2 to 59.4%, with a mean of 44%. The difference in solubility measurements may be the result of varying experimental procedures and technique, including sample particle size, bag pore size and washing technique. The hand-washing technique used in this experiment may have produced different solubility results when compared to other methods which use mechanical washing procedures.

Pore size of the rumen incubation bags may have more influence on DM solubility. Research that obtained considerably higher DM solubility values, Khorasani et al. (2000) and Ramsey et al. (2001) used bags with pore size of 57.5µm and 50µm, respectively; in comparison to the 41µm pore size of the bags used in this trial.

In this study, both rolled and ground treatments of Valier were lower ( $P<0.05$ ) in the solubility of DM, CP and starch than the other varieties. This resulted in lower ( $P<0.05$ ) EDDM and EDCP for Harrington and Valier than for CDC Bold and CDC Dolly (Table 2.4.). Even with the smaller particle size of ground samples, the observed variation in rate and extent of digestion indicates that each barley variety possesses a distinct physical structure and chemical composition which is involved in determining the rate and extent of physical degradation and digestion in the rumen, and gives each variety its own unique degradation kinetics.

In general, there was a significant variety by processing interaction ( $P<0.05$ ) on the rate of DM, CP and starch degradability observed among the four barley varieties included in this study. The nature of this interaction tended to be that grinding caused an increase in the rate and extent of digestibility for all varieties, but to a greater extent for CDC Bold and CDC Dolly compared to Harrington and Valier.

Table 2.5 shows the rumen degradation kinetics of four barley varieties for rolled samples only. As with the corn vs Harrington barley trial, the objective was to isolate the effect of barley variety on *in situ* rumen degradation characteristics by analyzing variables at a single level of processing. Rolling was chosen as it is the most common form of processing for barley in ruminant rations.

Statistical differences were observed in the rate of rumen disappearance ( $K_d$ ) of DM from ground samples, ranging from 38.4 and 39.9%  $h^{-1}$  for CDC Bold and CDC Dolly, respectively, to 28.4 and 29.3%  $h^{-1}$ , respectively, for Harrington and Valier. Despite the differences observed among the DM  $K_d$  of ground treatments, the rate of DM degradation for rolled treatments ranged from 13.7 to 16.6%  $h^{-1}$ , and showed no statistical difference between varieties, similar to the observation of Ramsey et al. (2001; 6.0 to 16.0%  $h^{-1}$ ). Neither study was able to detect any statistical difference in the rates of DM and starch degradation between different varieties of rolled barley. Similarly, Yu et al. (2003) did not find any differences in the rate of DM, CP or starch degradation between samples of rolled Harrington and Valier barley. However, in the study by Yu et al. (2003) as well as the present experiment, it should be noted that numerically, Valier had the lowest rate of digestion ( $K_d$ ) for DM and starch. Valier was also statistically lower ( $P < 0.05$ ) than the other three barley varieties for EDDM, EDCP and EDSt (Table 2.5). Significant differences ( $P < 0.05$ ) were detected in the soluble fraction of DM, CP and starch between rolled barley samples. Valier had lower ( $P < 0.05$ ) solubility of DM, CP and starch than both CDC Bold and CDC Dolly, and was similar to Harrington for CP and starch solubility (Table 2.5). The relationship between Harrington and Valier barley for rumen degradation kinetics observed in the present study confirmed previous work conducted by Yu et al. (2003). Both studies found Harrington to be numerically, but not statistically ( $P > 0.05$ ), higher than Valier for rate of DM and starch rumen degradation ( $K_d$ ), and significantly higher ( $P < 0.05$ ) in S (DM and CP), EDDM and EDCP.

Although the rate of DM, CP and starch degradation measured in this experiment showed no statistical differences among the four barley varieties ( $P = 0.255$ , 0.195 and 0.299, respectively), the numerical order was the same as that of the ground samples with CDC Bold showing the fastest  $K_d$  values followed by CDC Dolly,

**Table 2.5.** Trial 2 *in situ* rumen degradation kinetics for rolled samples of CDC Bold, CDC Dolly, Harrington and Valier barley.

| Item                                                      | Barley variety |          |            |         | SEM   | P-value |
|-----------------------------------------------------------|----------------|----------|------------|---------|-------|---------|
|                                                           | Bold           | Dolly    | Harrington | Valier  |       |         |
| <i>Rumen degradation characteristics of dry matter</i>    |                |          |            |         |       |         |
| Kd (%/h)                                                  | 16.61          | 15.77    | 14.92      | 13.71   | 0.985 | 0.2555  |
| S (%)                                                     | 10.50 a        | 9.25 a   | 9.42 a     | 6.68 b  | 0.642 | 0.0030  |
| D (%)                                                     | 70.48          | 71.75    | 70.23      | 69.67   | 1.368 | 0.6765  |
| EDDM (%)                                                  | 62.20 a        | 61.01 a  | 59.30 a    | 55.01 b | 1.154 | 0.0022  |
| <i>Rumen degradation characteristics of crude protein</i> |                |          |            |         |       |         |
| Kd (%/h)                                                  | 12.24          | 11.77    | 10.11      | 10.15   | 0.799 | 0.1953  |
| S (%)                                                     | 7.04 a         | 4.71 ab  | 2.75 bc    | 0.53 c  | 0.872 | 0.0013  |
| D (%)                                                     | 77.79          | 79.64    | 78.73      | 78.09   | 2.068 | 0.8661  |
| EDCP (%)                                                  | 59.16 a        | 57.19 a  | 52.01 b    | 48.49 c | 1.327 | 0.0001  |
| <i>Rumen degradation characteristics of starch</i>        |                |          |            |         |       |         |
| Kd (%/h)                                                  | 16.98          | 16.56    | 16.67      | 14.76   | 0.860 | 0.2994  |
| S (%)                                                     | 11.39 a        | 8.40 ab  | 6.84 bc    | 4.01 c  | 1.252 | 0.0142  |
| D (%)                                                     | 81.85          | 85.15    | 83.46      | 85.25   | 1.635 | 0.3919  |
| EDSt (%)                                                  | 71.86 a        | 70.76 ab | 68.00 b    | 64.44 c | 1.323 | 0.0048  |

Means with different letters in the same row are significantly different ( $P < 0.05$ )

Harrington and Valier (Table 2.5). Valier was statistically lower ( $P < 0.05$ ) in EDDM, EDCP, EDSt and S of DM than the other three varieties (Table 2.5), indicating that Valier barley has less extensive rumen fermentation than the other barley varieties even though the actual rate of degradation was statistically similar.

Despite the lack of significant differences in  $K_d$ , the ranking of barley varieties in the order of decreasing  $K_d$  corresponded to the order of decreasing starch:protein ratio in the kernel as estimated by chemical analysis (Table 2.1) (CDC Bold > CDC Dolly > Harrington > Valier). This observation helps to support the theory that a lower starch:protein ratio is indicative of lower DM degradation in the rumen. The assumption is that higher protein content, which is reflected in a lower starch:protein ratio, gives greater 'protection' to starch granules in the endosperm and reduces the rate of rumen degradation by hindering the access of rumen microbes to the starch granules.

Besides rate of rumen DM degradation, most of the rumen degradation kinetics (with few exceptions, i.e.  $K_d$  of starch), were consistently ranked in the same order: CDC Bold, CDC Dolly, Harrington and Valier, with CDC Bold having the highest, and Valier the lowest values for  $K_d$ , of DM and CP, S and EDDM; for starch: S and EDSt.

### **Conclusions In situ Results**

The results of the *in situ* digestibility show that the rate and extent of rumen disappearance varies both between cereal grain types (ie. corn and barley) as well as among varieties of barley. Corn exhibited slower rates of *in situ* DM, CP and starch degradation than barley at two different levels of processing (rolling and grinding), confirming previous research which indicated that the availability of corn starch for rumen fermentation processes is less than that of barley. Although fine processing (grinding) served to increase the rate and extent of digestion for both corn and barley, the magnitude of the response was differentially affected. Ground Harrington (GH) showed a significant increase in rate and extent of DM degradation over the rolled form, while there was no significant difference ( $P < 0.05$ ) detected in the rate of degradation between rolled and ground corn (Table 2.2.). This indicates that the physical structure of the grain kernel is not the only factor which influences digestibility. Chemical

constituents and molecular structure must also be involved in the total determination of DM, CP and starch digestion.

*In situ* digestibility comparison of four different varieties of barley also revealed differences in the rate and extent of DM, CP and starch digestion. Valier consistently proved to be less extensively degraded and at a slower rate than the other three varieties, verifying previous reports of slower DM degradability than other barley varieties (Yu et al., 2003).

The starch:protein ratio calculated from total starch and CP analysis on whole kernel samples of each grain was also of interest in this study. From our experimental observations, it seems that a lower starch:protein ratio is related to reduced rate and extent of rumen degradation measured by *in situ* methods.

With respect to ruminant feeding programs, favorable digestive characteristics, such as a slower rate of ruminal starch degradation, could become a criterion for selection in barley breeding programs. Such selection and breeding programs with the specific goal of developing varieties of feed barley that exemplify the desirable digestibility rates and characteristics for ruminants would benefit from a technique that would provide rapid analysis and evaluation of barley grain varieties for these desirable characteristics.

These findings indicate that there is potential for further investigation into the molecular and biochemical nature of cereal grains and their relationship to digestion within the rumen. Physical factors such as kernel structure and degree of processing which affect digestibility can be evaluated using *in situ* techniques, but physical factors represent only one element of the total mechanism involved in cereal grain digestion. Based on the known physical digestibility, further research would be valuable to explore the use of other technologies that may help to clarify the biochemical and molecular factors which are also important contributors to the overall digestibility of cereal grains.

### 3.0 Global and Synchrotron Infrared Microspectroscopy

Considerable potential exists for variation in size and weight among grain kernels within a population. Variation exists between kernels originating from different plants, between kernels from different tillers on the same plant, and between kernels from different locations on the same spikelet. Therefore, since the sample size for this experiment was limited (5 kernels from each variety), selecting a random sample was not possible because the small sample size would not necessarily provide an accurate representation of the entire population. As a result, seed selection was intentionally biased in order to obtain a uniform group of kernels for analysis. The intent was to avoid selecting extreme observations that would significantly skew the data due to the small sample size. A sub-sample of the screened portion from each barley variety used for *in situ* experimental work was further selected by weighing out individual kernels. This was done to obtain a small pool of kernels with the same weight and density. Valier was lighter and less plump than the other three varieties, likely as a result of different weather and soil conditions during the growing season.

The complex lower region of the mid-infrared spectrum between 1800-950  $\text{cm}^{-1}$  is known as the 'fingerprint region' as nearly every organic compound produces a unique pattern of absorbance in this area. A single representative FTIRM spectrum, collected using global-source IR light, from each of the four barley varieties is shown in Figure 3.3. Each spectrum represents a single pixel, randomly chosen from the 250 taken from each variety, and is meant to show the typical absorptions present in the 1750-900  $\text{cm}^{-1}$  region of the barley spectrum. Two predominant features exist in the IR spectrum relating to protein and are seen in all four of the barley spectra. An absorption band assigned to protein Amide I is centered at 1650  $\text{cm}^{-1}$  and is attributed to the stretching vibration of C=O bonds in the protein backbone (Wetzel et al., 1998; Miller, 2004). Amide II is represented by the weaker absorption band at approximately 1550  $\text{cm}^{-1}$ , and is characteristic of bending vibrations of the N-H bonds coupled with C-N stretching (Jackson and Mantsch, 2000). The Amide II absorption is the product of complex vibrations involving several different functional groups, therefore it is less useful for estimating protein content than Amide I (Colthup et al., 1990).

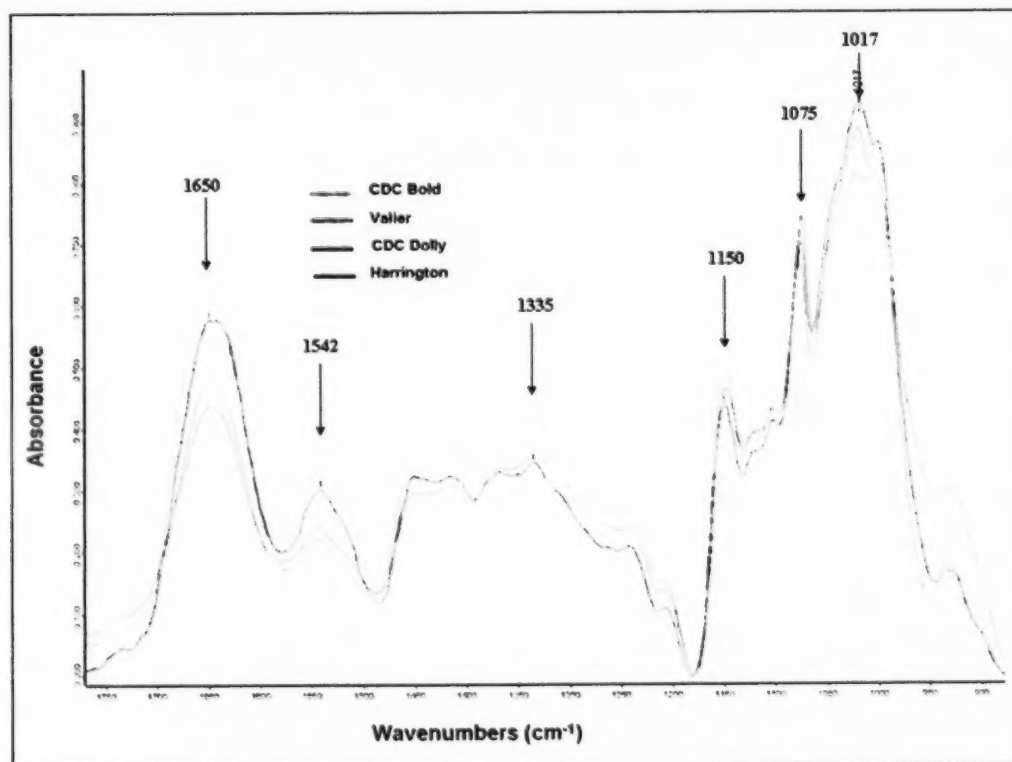


Figure 3.3. Enlarged 1850-800  $\text{cm}^{-1}$  region of FTIR spectra of one random spot sample from each of Harrington, CDC Bold, CDC Dolly and Valier barley endosperm tissue obtained with global source IR light. Harrington absorption peaks are evident at 1650, 1542, 1335, 1150, 1075 and 1017  $\text{cm}^{-1}$ .

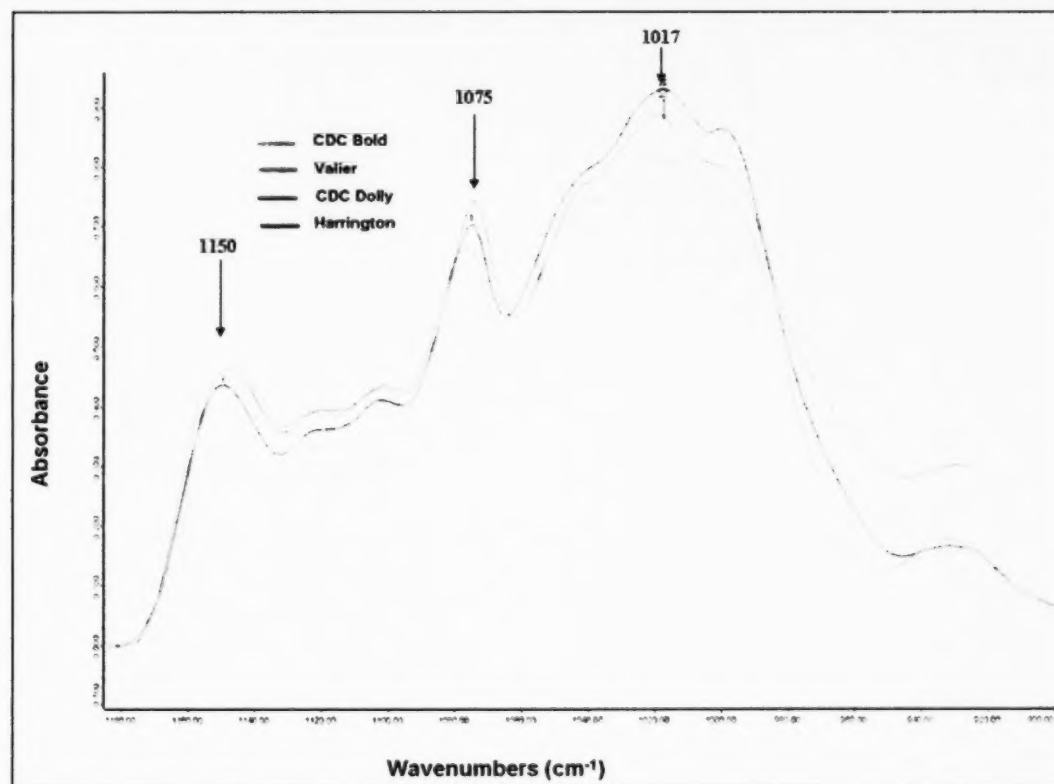


Figure 3.4. Enlarged image of the CHO region ( $1200\text{--}900\text{ cm}^{-1}$ ) region of the FTIR spectra of one random spot sample from Harrington, CDC Bold, CDC Dolly and Valier barley endosperm tissue obtained with globar source IR light. Harrington CHO peaks are evident at  $1150$ ,  $1075$  and  $1017\text{ cm}^{-1}$ .

Figure 3.4 shows an enlarged image of the 1200-900  $\text{cm}^{-1}$  region typically occupied by various OH and CO bond absorptions from both structural (cellulosic) and non-structural (starch-type) polysaccharides (Yu et al., 2004b). The spectral region containing carbohydrate absorption assignments is complex and not fully understood. There are three prominent absorptions consistently seen in the spectra of barley and corn endosperm tissue. The first, centered at 1150  $\text{cm}^{-1}$ , is attributed to the C-O-C stretching vibrations of glycosidic linkages (Kačuráková and Wilson, 2001; Robert et al., 2005; Philippe et al., 2006). This is a fairly distinct and sharp absorption common to all four barley spectra, as is the absorption peak centered at 1075  $\text{cm}^{-1}$ . This absorption likely arises from C-O-H bending vibrations of polysaccharide-type compounds (Cael et al., 1973), but the exact nature of the functional group responsible for this particular peak is undetermined. The largest of the three carbohydrate peaks seen in the absorption spectrum is centered at approximately 1017  $\text{cm}^{-1}$ . Non-structural carbohydrate such as starch is most often attributed to absorption at 1025  $\text{cm}^{-1}$  (Wetzel et al., 1998, Yu et al., 2004b), however, there appears to be no distinct peak at this location evident in these spectra.

The third major CHO absorption peak (1065-950  $\text{cm}^{-1}$ ) in these spectra is very broad and has noticeable shoulders on either side of the main peak. This could be a result of the larger aperture size of the global light source used for this experiment. Since the 50  $\mu\text{m}$  aperture encompasses an area that is larger than the dimension of starch granules (10-25  $\mu\text{m}$ ) (MacGregor and Fincher, 1993), the spectra may reflect the presence of other carbohydrates such as cell wall components (arabinoxylans). It may also be an indication of the heterogeneous nature of the carbohydrates present in the endosperm tissue.

### 3.3.1 Global-source IR Microspectroscopy

Least square means of peak area for Amide I and CHO (starch), and ratios of CHO to Amide I are shown in Table 3.6. Data from this table was obtained from individual spot sample spectra taken from 50 randomly selected points within the endosperm region from five different sections of each grain (corn, CDC Bold, CDC Dolly, Harrington and Valier) for a total of 250 spectra per grain variety. Discretion must be used when

evaluating the absorbance area measurements for the Amide I and CHO peaks. Direct comparison is difficult because they are not absolute values, but rather represent the relative content of functional groups present at each sampling point. Absorbance units show the amount of IR energy absorbed by the sample, which is directly proportional to sample thickness. Direct comparison of the area under the absorbance peak between two or more discrete spectra does not account for changes in sample thickness between pixel locations. Differences in the IR absorbance that occur as a result of variation in tissue thickness can be accounted for by using peak height or area ratios (Miller, 2004). Therefore, the ratio of CHO:Amide I peak area is the most accurate and practical way of comparing spectra collected from various spatial locations on different tissue samples. There was no difference between Amide I ( $1710\text{-}1580\text{ cm}^{-1}$ ) peak area absorptions for corn and Harrington, possibly indicating similar protein content in the endosperm tissue of each grain. In contrast, the amount of protein measured by chemical analysis (Table 2.1) showed that Harrington had slightly higher protein content than corn (14.1 vs 11.5%, respectively), keeping in mind that this was an analysis of the entire kernel, rather than solely the endosperm region. The IR data in Table 3.6 indicates that the same is true of the endosperm tissue when it is isolated from other tissues and examined separately.

Carbohydrate absorption peak area centered at  $1017\text{ cm}^{-1}$  was significantly higher ( $P<0.05$ ) for corn than Harrington barley. Again, this was consistent with the chemical starch analysis in which corn showed higher starch content than Harrington based on whole kernel analysis; however, there was a noticeable degree of difference in the magnitude of starch content measured using the two different techniques (FTIRM and chemical analysis).

Starch content determined by the total starch assay (Table 2.1) showed that corn was only slightly higher in starch content than Harrington barley (66.5 vs 57.8%, respectively), while the IR measurements of CHO peak area for corn are nearly double that of barley (Table 3.6). Because the chemical analysis was a reflection of the starch content in the entire kernel whereas the IR technique was measuring starch in endosperm tissue only, this may indicate differences in the distribution of starch throughout the kernel.

Table 3.6. CLS global light source spot sample data: LS Means of peak area for Amide I and CHO, and ratio of CHO to Amide I for corn and Harrington.

| Variety              | LS means (Absorbance units) <sup>z</sup> |                                   |                                   |
|----------------------|------------------------------------------|-----------------------------------|-----------------------------------|
|                      | Amide I                                  | CHO                               | CHO:Amide I                       |
| Harrington           | 24.35 ( $\pm$ 7.56)                      | 22.03 ( $\pm$ 6.63) <sup>b</sup>  | 0.993 ( $\pm$ 0.431) <sup>b</sup> |
| Corn                 | 23.96 ( $\pm$ 7.53)                      | 43.67 ( $\pm$ 10.56) <sup>a</sup> | 1.964 ( $\pm$ 0.732) <sup>a</sup> |
| Pooled SEM           | 0.526                                    | 0.598                             | 0.036                             |
| P-value <sup>y</sup> | 0.5998                                   | <0.0001                           | <0.0001                           |

Means with different letters in the same column are significantly different ( $P < 0.05$ )

<sup>z</sup> Represents the mean of spot sample spectra (n=26 to 46) from five different tissue sections of each grain

<sup>y</sup> P-value indicates effect of variety

The amount of starch observed in the endosperm tissue using IR analysis was much greater for corn, possibly indicating that starch is proportionally higher in the endosperm tissue of corn compared to barley. Since these peak area measurements do not take tissue thickness into consideration, the numerical difference between them is not necessarily an absolute value, and may reflect some inconsistency in the texture and thickness of the tissue sections. We are also making the assumption that the CHO absorption peak centered at  $1017\text{ cm}^{-1}$  is attributed solely to starch. This assumption is based on the knowledge that the majority of the carbohydrate in the endosperm region is starch (Kent and Evers, 1994), and that only minor portions of the total carbohydrate are other forms (such as cell wall or structural components).

Because of the lack of certainty for estimating the absolute amount of chemical constituents in a sample and the difficulties in making a comparison of peak absorption measurements between two different samples, FTIRM may not be the most appropriate technique to use if quantitative measurement of a particular functional group is required. However, it does appear to be useful for preliminary analysis of samples for the purpose of ranking them in order of increasing/decreasing content of a particular constituent of interest.

Due to the limitations of comparing peak absorbance of single components, the ratio of absorption values is of more interest and diagnostic value. The advantage of this method is that by using ratios, any variation in spectral intensity resulting from differences in tissue thickness is eliminated (Yu et al, 2004b). Therefore, the CHO:Amide I ratio was calculated and analyzed in these experiments. The ratio of CHO:Amide I peak area absorption is higher ( $P<0.05$ ) for corn than Harrington barley. Based on the higher starch absorption of corn, this is consistent with the higher starch:protein ratio observed from the chemical analysis of corn (Table 2.1). Although there appears to be consistency between the two methods, the results do not agree with the observations from *in situ* rumen degradation trial for the same grains where corn showed both a lower rate and extent of DM degradation than corn. McAllister et al. (1993) suggested that the protein matrix surrounding starch granules in the endosperm is a major factor responsible for differences in the rate of rumen starch digestion of cereal grains. The structure of the protein matrix provides a certain amount of

'protection' to starch granules, shielding them from enzymatic attack of rumen microorganisms. Assuming that more protein would increase this level of protection, it follows that a lower starch:protein ratio (ie. more protein per unit of starch) would tend to reduce the rate of rumen degradation. However, when comparing the *in situ* rumen degradation of corn and Harrington barley with the starch:protein ratio determined by both chemical and IR techniques, it appears that these two grains behave in the exact opposite manner to what would be predicted based on the starch:protein ratio. This would indicate that it may be the nature or composition of the protein rather than the quantity which serves to 'protect' the starch granules from digestion and slows the rate and extent of starch degradation by shielding the starch granules from microbial attack. For the purpose of classification, cereal proteins are divided into four major groups based on their solubilities: albumins, globulins, prolamins and glutelins. Differences in the amount of each protein fraction in corn and barley exist. Albumins and globulins, soluble in water and dilute salt solutions, respectively, comprise approximately 25% of the total protein content of barley, but only account for 6% of corn protein (Lookhart, 1991). In addition, glutelins, which are soluble in acid or alkali, account for nearly 20% more of the total protein content in corn than barley. Finally, the major storage protein fraction in corn, the prolamins, is called zein whereas prolamins present in barley are hordeins (Lookhart, 1991). Differences in the amino acid content and structure that exist between the proteins of different grain types (ie. corn and barley) may influence how polypeptides react to the rumen degradation processes, contributing to the observed differences in starch degradability in corn and barley. Corn is more slowly digested even though there is relatively less protein present in the entire corn kernel and in the endosperm tissue.

Table 3.7 presents the least square means comparison of peak area from spot sample spectra taken from the endosperm region of CDC Bold, CDC Dolly, Harrington and Valier barley. The values in Table 3.7 represent the mean peak areas from spectra (n=26 to 46) collected from each of five different sections of tissue from each barley variety. Evidently, there is a wide variation in the absorption intensity of Amide I and CHO even among spectra from the same tissue section. For example, the peak area absorbance of CHO and Amide I from 46 spectra taken from a single section of

Harrington barley ranged from 8.7 to 38.2 and 7.7 to 51.7, respectively (data not shown), indicating the heterogeneous nature of the protein and starch distribution within the endosperm tissue. Differences in the absorbance of Amide I and CHO seen between varieties, however, are not necessarily indicative of the difference in relative amount of chemical components.

Harrington had higher ( $P < 0.05$ ) absorbance intensity for CHO and lower absorbance intensity for Amide I than Valier (22.0 vs. 19.2, and 24.4 vs. 28.1, respectively). Similarly, Yu et al. (2004a) observed that Valier tended to have lower starch and higher protein absorbance intensity than Harrington.

CDC Bold had the largest CHO:Amide I ratio (1.109), followed by CDC Dolly and Harrington (.947 and .993, respectively) and Valier (.767). Yu et al. (2004a) also found that Harrington had greater ( $P < 0.05$ ) ratio of starch to protein than Valier. This order follows the same ordinal ranking as the starch:protein ratio determined by chemical analysis (Table 2.1). More importantly, it is also consistent with the results of the *in situ* rumen degradation kinetics reported in Chapter 2 where CDC Bold had the fastest rate of rumen degradation, followed by CDC Dolly, Harrington and Valier (Table 2.4). Yu et al. (2004a) suggested that a greater association of the protein matrix with starch granules in the endosperm of barley may serve to limit the access of ruminal microorganisms to starch granules, thus reducing the rate and extent of rumen degradation. From this suggestion as well as observations of the current study, a lower starch to protein ratio in barley seems to be associated with a slower rate of rumen degradation. This hypothesis holds true when comparing cereal grains within the same family, but does not, however, appear to apply when comparing grains from different families (ie. barley varieties can be compared, but not barley to corn).

Table 3.7. CLS global light source spot sample data: peak area comparison of CHO and Amide I of CDC Bold, CDC Dolly, Harrington and Valier barley.

| Variety              | LS means (Absorbance units) <sup>z</sup> |                                  |                                   |
|----------------------|------------------------------------------|----------------------------------|-----------------------------------|
|                      | Amide I                                  | CHO                              | CHO:Amide I                       |
| Bold                 | 20.62 ( $\pm$ 8.10) <sup>c</sup>         | 20.57 ( $\pm$ 6.87) <sup>b</sup> | 1.109 ( $\pm$ 0.481) <sup>a</sup> |
| Dolly                | 25.82 ( $\pm$ 8.27) <sup>b</sup>         | 22.43 ( $\pm$ 7.72) <sup>a</sup> | 0.947 ( $\pm$ 0.394) <sup>b</sup> |
| Harrington           | 24.35 ( $\pm$ 7.56) <sup>b</sup>         | 22.03 ( $\pm$ 6.63) <sup>a</sup> | 0.993 ( $\pm$ 0.431) <sup>b</sup> |
| Valier <sup>y</sup>  | 28.11 ( $\pm$ 9.83) <sup>a</sup>         | 19.21 ( $\pm$ 7.08) <sup>b</sup> | 0.767 ( $\pm$ 0.378) <sup>c</sup> |
| Pooled SEM           | 0.595                                    | 0.521                            | 0.031                             |
| P-value <sup>x</sup> | <0.0001                                  | <0.0001                          | <0.0001                           |

Means with different letters in the same column are significantly different (P<0.05)

<sup>z</sup> Represents the mean of spot sample spectra (n=26 to 46) from five different tissue sections of each grain

<sup>y</sup> LS mean obtained from spectra collected from four tissue sections

<sup>x</sup>P-value indicates effect of variety

From this experiment, it seems that analysis with global-source FTIRM is able to rank barley varieties according to the rate of rumen degradability based on measurement of the CHO to Amide I ratio. However, this observation is based on limited data from only four barley varieties compared with a single corn sample. To more accurately test this hypothesis, IR analysis for other cereal grains such as wheat and oat would be useful for comparison with the corn and barley results from this study. It would also be beneficial to increase the number of corn samples in order to observe variation among different varieties of corn as seen with the barley varieties in this experiment.

### **3.3.2 Synchrotron-source IR Microspectroscopy: Reflectance Mode**

Synchrotron FTIRM analysis of corn, CDC Bold, CDC Dolly, Harrington and Valier was conducted at ANKA. Initially, the experimental protocol was to analyze the exact same 50 spot samples that were collected with the global IR light source at the CLS by uploading saved X,Y coordinates from each point into the OPUS software program at ANKA. The objective was to directly compare the spectra obtained with synchrotron and global-source IR light to determine the differences between their analytical and functional capabilities by removing the variable of tissue variation. However, the microscope configuration used at ANKA did not have the same stage reference points and dimensions as the instrument used at the CLS. Consequently, the saved coordinates did not correspond with the same point on the microscope slides, so new spot sample points were randomly chosen.

Figure 3.5 shows a visual example of the quality of the spectral data collected in the ANKA experiment. Many of the spectra collected showed high absorbance values in the OH stretch region ( $3100\text{--}3600\text{ cm}^{-1}$ ) and the carbohydrate region ( $1200\text{--}900\text{ cm}^{-1}$ ) with some detector saturation. Spectra also showed poor signal to noise ratio (S/N) indicating that the tissue was too thick for optimal analysis. Waves and oscillations were seen in the baseline of many of the ANKA spectra (between  $2800\text{--}2000\text{ cm}^{-1}$ ), likely due to the fact that portions of the tissue sections were cracking and lifting off of the IR slides. Any inconsistencies on the surface of the tissue section, or

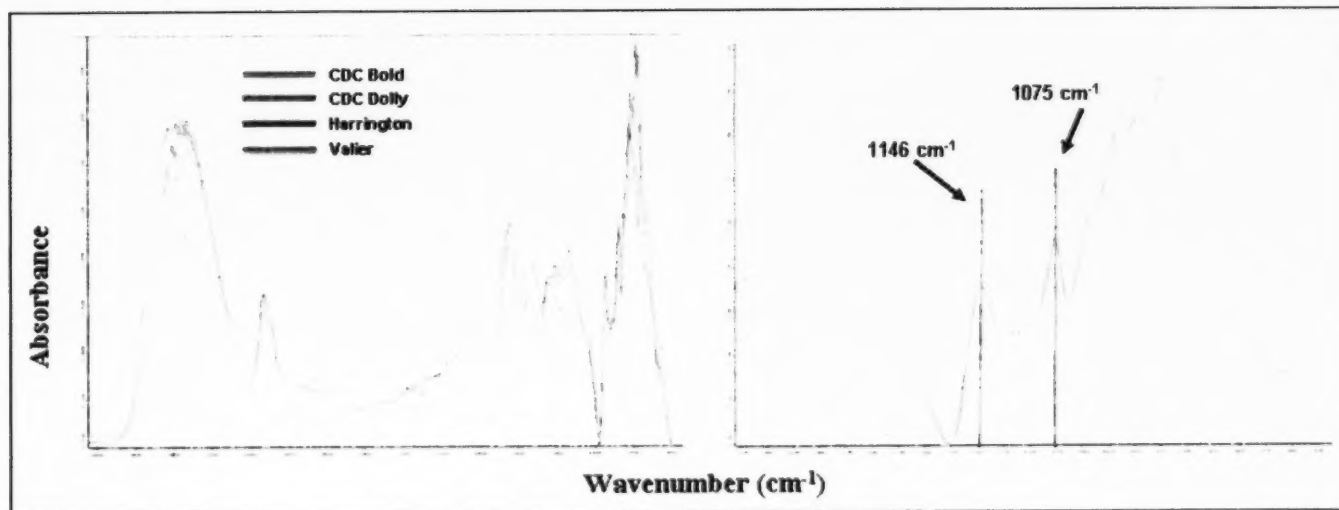


Figure 3.5. Spot sample spectra of four barley varieties obtained with synchrotron-source light at ANKA. A) Entire mid-IR spectral range showing oscillations in the baseline ( $2000\text{--}2600\text{ cm}^{-1}$ ) and high absorbance in the OH stretch ( $3000\text{--}3600\text{ cm}^{-1}$ ) and CHO ( $1200\text{--}900\text{ cm}^{-1}$ ) regions and B) Enlarged image of the  $1300\text{--}900\text{ cm}^{-1}$  region showing two common CHO absorptions at  $1146$  and  $1075\text{ cm}^{-1}$ .

space between the tissue and the IR slide can cause oscillations in the background of the IR spectra (Miller, 2004) and reduce the quality of the data.

Due to the poor quality of the spectral data collected at ANKA, these spectra were not used for direct analysis, but rather for qualitative evaluation. In a general assessment of the collected data, there does not appear to be a prominent absorption peak at  $1025\text{ cm}^{-1}$  which is commonly attributed to starch (Wetzel, 1998, Yu et al., 2004a). However, the two smaller CHO absorption peaks, located at approximately  $1146$  and  $1075\text{ cm}^{-1}$ , are consistently present in most of the spectra. These absorptions are consistent with the first and second carbohydrate peaks present in the typical IR spectrum of cereal grain tissue obtained with the global-source light. Although there are similarities in this data to the CLS global-source data, the poor spectral quality reduces its reliability and accuracy. Problems with structural integrity of the tissue sections as well as the use of reflectance mode for IR analysis were identified as the key factors contributing to the poor spectral quality.

Mathematical manipulations, such as second derivative calculation, smoothing, and Fourier transform self-deconvolution, can be applied to enhance the original spectral data. However, these techniques cannot improve the quality of the data, and extreme caution must be exercised when using any type of data manipulations. When starting with poor quality data, rather than assisting in elucidating information from the original data, these manipulations would essentially cause further distortion of any of the information contained in the original spectra.

### **3.3.3 Synchrotron-source IR Microspectroscopy: Transmission Mode**

#### **3.3.3.1 Chemical Imaging**

Figures 3.9 to 3.11 (A) show visual images of the tissue regions from which functional group maps were taken from corn, Harrington and Valier. Maps were positioned to include the various layers of tissue from the outer pericarp and seed coat, through the aleurone layer and into the endosperm region of the tissue. Single-pixel spectra were collected every  $12\mu\text{m}$  according to the aperture size of the microscope. The overlaid grid represents each individual pixel location where a spectrum was collected in order to generate the overall mapping area. Color images of the mapped

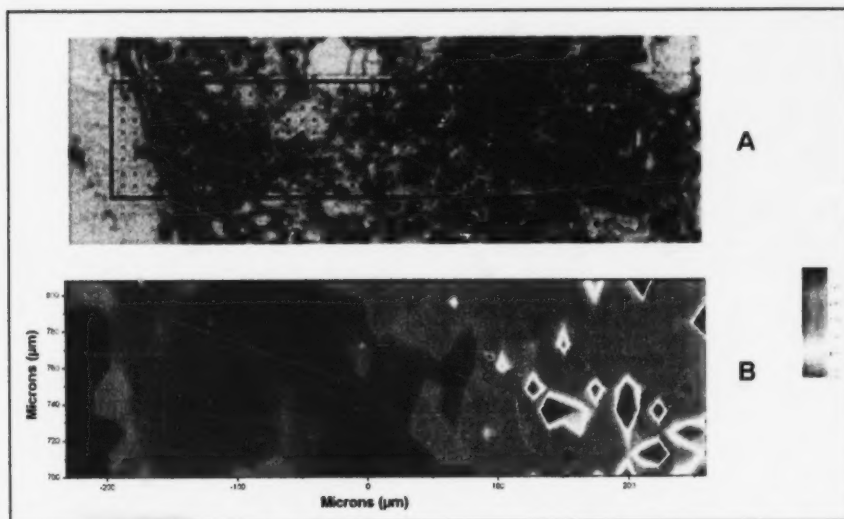


Figure 3.6. Visible image of Harrington barley with overlay of the mapping grid shown in blue. B) False color map showing the ratio of the area under the CHO (1065-950  $\text{cm}^{-1}$ ) and Amide I (1710-1580  $\text{cm}^{-1}$ ) peaks.

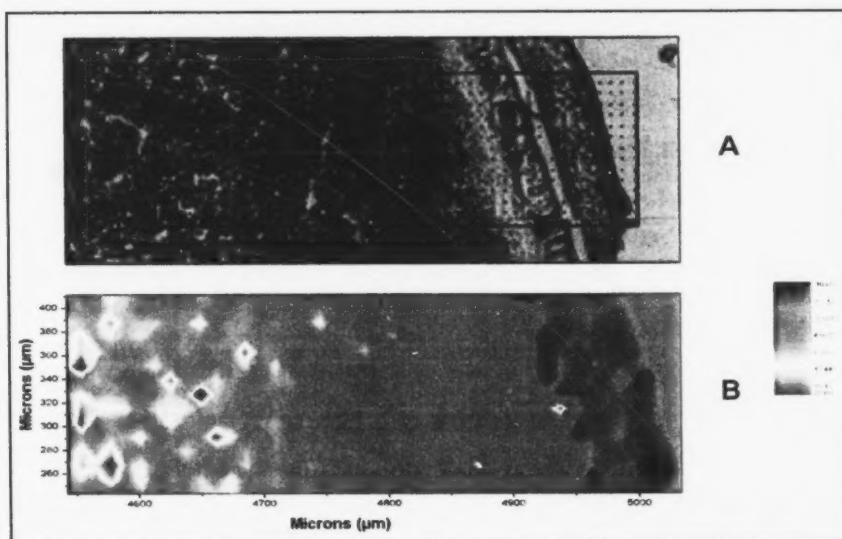


Figure 3.7. Visible image (A) of 39P78 corn with overlay of grid showing mapping points. B) False color chemical image depicting the ratio of CHO (1065-950  $\text{cm}^{-1}$ ) to Amide I (1710-1580  $\text{cm}^{-1}$ ) peak area absorption.

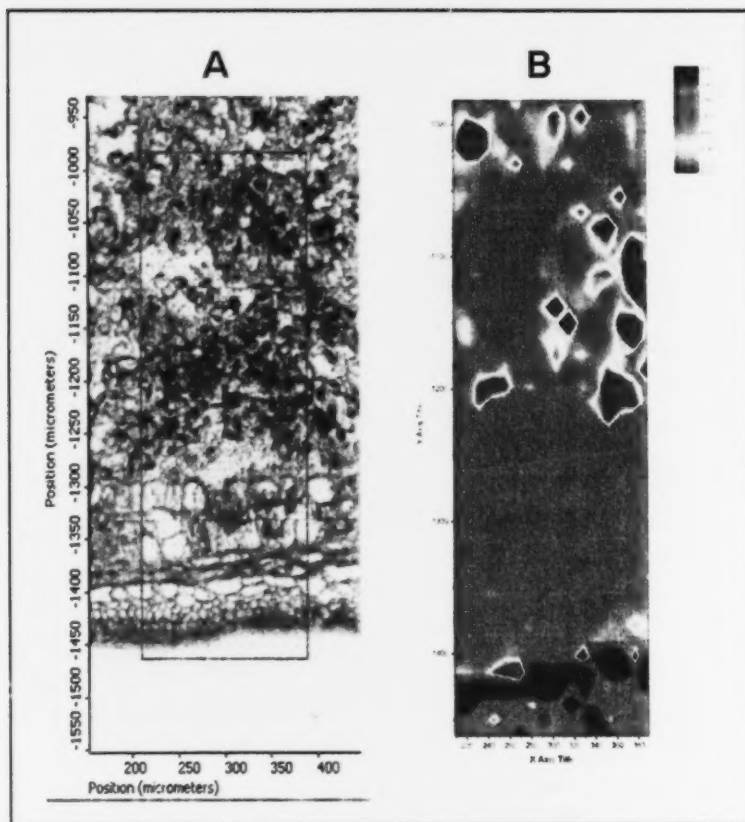


Figure 3.8. Visible image (A) of Valier barley with overlay of grid showing mapping points. B) False color chemical image of the ratio of CHO ( $1065-950\text{ cm}^{-1}$ ) to Amide I ( $1710-1580\text{ cm}^{-1}$ ) peak area absorption.

region of tissue show the spatial; distribution and intensity of the ratio of CHO to Amide I peak area absorptions, where higher intensity represents a larger content of starch relative to protein. Red represents regions of highest intensity while green/blue represent the lowest absorption intensity. Areas of black on the false color maps represent flawed data points that were removed from the data matrix.

In all three grains, the chemical maps show variation in the distribution of functional groups within the tissue sampling area. Starch content is relatively low in the regions of pericarp, seed coat, and aleurone, but gradually increases into the starchy endosperm tissue. Groups of starch granules can be identified by the high absorption intensity spots in the endosperm region. Based on this data, care was taken in determining the location of the sampling region for spot sample data collection.

The value of chemical imaging is that it provides insight into the relative distribution of chemical constituents in various regions of the grain kernel. Such spatial information is lost in chemical analysis of the whole kernel, and the ability to isolate specific regions of the kernel (endosperm, cell wall, etc.) for individual analysis is impossible. Use of IR images offers the ability to relate the concentration of chemical constituents to anatomical structure. IR mapping may provide the knowledge to predict digestibility of CHO and other tissue components based on where they are located in the kernel relative to other components (ie. starch and protein, where there is more protein, starch will supposedly be less digestible)

#### **3.3.3.2 Spot sample analysis**

Due to poor results from the reflectance mode synchrotron analysis conducted at ANKA, a second synchrotron-source FTIRM experiment was performed to compare the results with global-source data previously collected at the CLS. Because the synchrotron-source analysis of samples in reflectance mode (carried out at ANKA) resulted in poor data with unsatisfactory spectral quality, further data using synchrotron-source IR light was collected in transmission mode. This required modifications to the sample preparation methodology, and use of BaF<sub>2</sub> spectral windows rather than IR reflectance slides.

Samples for this experimental procedure were again cut to 6 $\mu$ m thickness, but instead of sectioning frozen, untreated seeds, the samples were embedded in paraffin before

sectioning. The purpose of using a hydrocarbon matrix is to maintain the physical integrity of the sample during sectioning (Jackson and Mantsch, 2000). The method is a modified version of the general paraffin embedding process used for animal tissue at Prairie Diagnostic Services, Saskatoon, SK (Phil Dillman, personal communication). In the final step of the procedure, grain kernels are soaked in Paraplast for an extended period of time (38.5 hours) to allow the paraffin to penetrate the outer seed coat. Paraffinization helped to reduce the amount of shattering and tearing as the sections were cut, producing a more even and homogenous section and maintaining the integrity of the thin tissue. This was likely one of the problems with the dry-mounted tissue used in the reflectance mode experiments at ANKA, and was a contributing factor in the poor spectral quality. Paraffinization of the grain kernels for the NSLS experiment appeared to enhance adhesion of the tissue sections to the BaF<sub>2</sub> windows, helping them to remain flat on the slide, and preventing parts of the tissue from lifting off of the slide. This was important as an uneven sample surface can scatter the incident IR light, further reducing collection efficiency and causing artifacts in the IR spectrum (Miller, 2004). Concerns with using embedding agents, such as paraffin, have been raised in that it can actively absorb IR light and produce noticeable absorption bands in the sample spectrum. However, the most intense absorbance features of paraffin are limited to the C-H stretch region (2800-3000 cm<sup>-1</sup>) (Miller and Dumas, 2006), and do not overlap the IR absorption features of the sample that are of interest in this experiment: carbohydrate and protein absorption (between 1700-900 cm<sup>-1</sup>). Although there is some concern that the solvents used in the paraffinization process may extract lipid components from the sample (Mike Jackson, personal communication), such extraction would cause minimal change to the compositional profile of the barley tissue as lipid comprises only 2-4% of the whole grain, and only 1-3% of the endosperm tissue (Morrison, 1993). Furthermore, the lipid content of the grain kernels was not of primary interest in this experiment; rather, the main focus was on starch and protein within the endosperm tissue. Future experimental procedures whose objective it is to

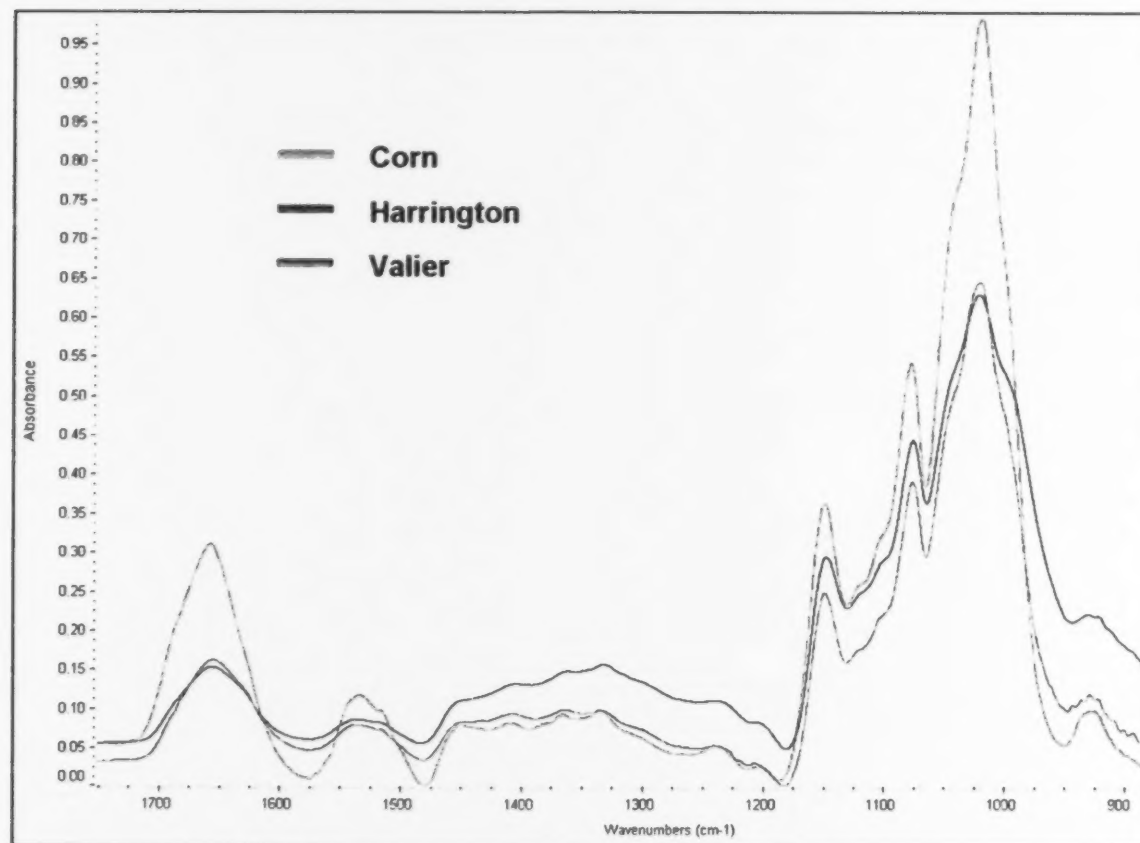


Figure 3.9. Enlarged 1750-900  $\text{cm}^{-1}$  region of FTIRM spectra of one random spot sample from Corn, Harrington and Valier endosperm tissue obtained with synchrotron-source IR light at NSLS. Harrington absorption peaks are evident at 1656, 1536, 1333, 1149, 1077 and 1025  $\text{cm}^{-1}$ .

examine lipids in barley grain may be required to employ different methods of sample preparation.

Due to experimental time limitations at the NSLS synchrotron facility, only three grain varieties were analyzed in this experiment. Corn and Harrington were chosen as they were used for direct comparison in the previous *in situ* digestibility and global-source IR experiments. Valier barley was chosen as the third variety based on its performance in the *in situ* digestibility trial in which it exhibited significantly different digestibility characteristics compared to the other three barley varieties.

Infrared spectra of corn, Harrington and Valier, obtained with a synchrotron light source and recorded in the  $1750\text{--}900\text{ cm}^{-1}$  region of the IR spectrum, are shown in Figure 3.9.

These three spectra are from a single pixel located in the endosperm region of their respective tissue sections. Although the general shape of all three spectra is similar, indicating the presence of similar functional groups, there are differences in the strength of absorption, as reflected in the variation in peak area and height. Corn shows stronger absorption peaks for Amide I ( $1656\text{ cm}^{-1}$ ) and the three CHO peaks ( $1150$ ,  $1077$ , and  $1025\text{ cm}^{-1}$ ), while Harrington and Valier are more similar in both line shape and degree of absorption, indicating that the endosperm tissue is more similar in nature between the barley varieties rather than the barley compared to corn. The corn spectrum had sharper, more defined Amide I absorption than the two barley varieties. The peak at  $1536\text{ cm}^{-1}$  is likely not a reflection of the Amide II absorption, which is typically centered at approximately  $1550\text{ cm}^{-1}$ , but rather a complex peak resulting in the overlap of several vibrational modes which obscure the relatively small Amide II absorption vibrations. The large absorptions in the CHO region may also contribute to the overlapping effect which overwhelms both the Amide I and II absorptions.

A smaller but consistent absorption peak at  $1333\text{ cm}^{-1}$  is also observed in spectra of all three grains. This absorption is rarely mentioned in previous reports of IR analysis of cereal grains, however, Cael et al. (1973) noted the presence of an absorption peak at  $1334\text{ cm}^{-1}$  in the Raman spectrum of amylose and  $\alpha$ -D-glucose which they assigned to a combination of the bending vibrations of C-O-H groups as well as a  $\text{CH}_2$ -related deformation mode.

Whereas barley spectra collected with global-source IR light exhibited a broad CHO absorption band centered at  $1017\text{ cm}^{-1}$ , the same CHO peak is shifted slightly to the right and centered at approximately  $1025\text{ cm}^{-1}$  in the synchrotron spectra (Figure 3.9). Similarly, prior synchrotron studies conducted on barley tissue, including our own experimental data collected at ANKA, detected a carbohydrate absorption peak attributed to starch centered at  $1025\text{ cm}^{-1}$  (Wetzel et al., 1998; Wetzel et al., 2003; Yu et al., 2004b), but did not report a peak existing at  $1017\text{ cm}^{-1}$ . There appears to be consistency in the location of third carbohydrate (starch) absorption peak at  $1025\text{ cm}^{-1}$  when comparing data collected using the same type of IR light source. The type of IR light source used for analysis may have an effect on the location where the third CHO peak is centered. However, this cannot be determined strictly from one experiment, and there is no available literature which includes barley analysis using global-source IR light with which to compare our experimental results. Further global-source IR analysis of several barley tissue samples would be required to confirm this observation.

Pixel size determined by the aperture setting may be responsible for the differences seen in the CHO absorption profile between the global and synchrotron data. As previously discussed, the  $50\text{ }\mu\text{m}$  aperture used for global data collection encompasses a larger area of tissue allowing for greater variation of functional groups that are present and able to absorb the incident light. On the other hand, the smaller  $12\text{ }\mu\text{m}$  aperture setting of the synchrotron experiment serves to limit the area of tissue being analyzed, and may serve to make the selected pixel more homogeneous.

Peak area measurements and ratios from spot sample spectra collected using synchrotron source IR light at the NSLS for corn and Harrington barley are shown in Table 3.8. Similar to the CLS global data (Table 3.6), CHO absorbance for corn was more than 65% greater than for Harrington ( $P < 0.05$ ), however, as previously discussed, this difference may be partially due to differences in sample tissue thickness and can not be taken as an absolute difference in concentration. There does, however, seem to be a certain degree of continuity in the CHO and Amide I peak absorption measurements obtained with the global and synchrotron-source analysis.

Table 3.8. NSLS synchrotron data: Comparison of least square means of peak area for Amide I and CHO, and ratio of CHO to Amide I for corn and Harrington barley.

| Variety              | LS Means (Absorbance units) <sup>z</sup> |                                   |                                 |
|----------------------|------------------------------------------|-----------------------------------|---------------------------------|
|                      | Amide I <sup>y</sup>                     | CHO <sup>x</sup>                  | CHO:Amide I                     |
| Harrington           | 6.34 ( $\pm$ 3.91) <sup>b</sup>          | 30.81 ( $\pm$ 13.09) <sup>b</sup> | 6.68 ( $\pm$ 5.51) <sup>b</sup> |
| Corn                 | 8.56 ( $\pm$ 4.46) <sup>a</sup>          | 50.98 ( $\pm$ 13.42) <sup>a</sup> | 8.09 ( $\pm$ 4.98) <sup>a</sup> |
| SEM                  | 0.293                                    | 1.005                             | 0.384                           |
| P-value <sup>w</sup> | <0.0001                                  | <0.0001                           | <0.0001                         |

Values in the same column with different letters are significantly different ( $P < 0.05$ )

<sup>z</sup> Mean of spot sample spectra ( $n=30$ ) from five different tissue sections for each grain variety. Total  $n$  for each grain = 150.

<sup>y</sup> Amide I peak area measured between 1710 to 1581  $\text{cm}^{-1}$ .

<sup>x</sup> CHO peak area measured between 1066 and 950  $\text{cm}^{-1}$

<sup>w</sup> P-value represents effect of variety

Peak area absorbance for Amide I was greater for corn than Harrington (8.56 vs 6.34, respectively), in contrast to the CLS global data in which Harrington had similar Amide I absorbance to corn. In addition, the magnitude of Amide I absorption is noticeably different between spectra collected with global and synchrotron source IR light. Global spectra have Amide I absorptions in the range of 23 to 24 absorbance units (Table 3.6) while synchrotron source spectra are in the range of 6.3 to 6.5 absorbance units (Table 3.8). This reduction in Amide I absorption intensity, paired with relatively consistent CHO absorption between the two methods results in the synchrotron data having much higher CHO to Amide I ratios than the corresponding global light source data. These differences in the relative absorbance values are likely due to the fact that the microscope aperture size used during spectral data collection changes from 50 $\mu$ m for global technique to 12 $\mu$ m for synchrotron work. Individual pixel size is decreased in the synchrotron data, making it easier to focus on a single tissue component, such as an individual starch granule, while excluding unwanted areas around the region of interest. The larger 50 $\mu$ m aperture used for global data collection encompasses a larger area which includes more protein contained in the matrix between starch granules. Alternatively, the smaller aperture setting utilized with the synchrotron is closer to cellular dimensions and can be focused more specifically on regions of tissue, such as single starch granules, excluding other material surrounding it. As a result, the synchrotron spectra show a large proportion of CHO which nearly overwhelms the protein (amide) absorption all together. Figure 3.10 shows the comparison of two spectra taken from Harrington barley using both global and synchrotron light sources. The synchrotron spectrum in red shows much higher absorptions in the CHO region, and less in the Amide I region when compared with the spectrum collected using the global light source.

Corn consistently showed a higher starch to protein ratio than Harrington barley when analyzed by total starch assay (Table 2.1), global IR (Table 3.6) and synchrotron IR (Table 3.8). Yet despite this, it exhibited the slowest rate of rumen degradation when evaluated using the *in situ* nylon bag technique. This disparity could be the result of differences in the structure of corn protein, which influences its ability to effectively shield starch granules from the digestive process. Rather than the quantity of protein

relative to starch, it may be that protein structure is more important to its ability to protect starch granules from digestion. Differences in the composition of the total protein content of corn and barley exist. For example, barley contains a larger proportion of albumins and globulins, and less glutelins than corn. The proportion of prolamines present in the two grains is relatively similar, but the type of prolamine differs between corn and barley. Zein proteins present in corn may exhibit different degradation characteristic from the hordeins found in barley due to variation in amino acid content and structure.

A direct comparison of peak area measurements obtained from synchrotron-source FTIRM for Harrington and Valier barley is presented in Table 3.9. The two barley varieties were analyzed separately from the corn in order to make the comparison consistent with previous analysis of *in situ* digestibility data and global-source IR data in which the barley varieties were grouped separately. Mean peak area measurements for Amide I and CHO did not differ between Harrington and Valier ( $P>0.05$ ); nor was there any statistical difference in the ratio of carbohydrate to Amide I (6.68 vs. 6.26, respectively). Numerically, Harrington had a higher carbohydrate to Amide I ratio than Valier, consistent with the observations of synchrotron-source FTIRM analysis by Yu et al. (2004b) as well as global-source data from this project (Table 3.7). Based on results from the *in situ* trials, Harrington and Valier tended to be the most similar in their digestibility characteristics, so it is not completely unexpected that no significant differences are seen in the IR peak area absorbencies of Amide I and CHO. Increasing the scope of the analysis by including additional barley varieties, particularly varieties such as CDC Bold and CDC Dolly which showed faster and greater *in situ* rumen degradation characteristics, would be of interest and could lead to observable differences in CHO to Amide I ratio as determined by synchrotron-source FTIRM. A consistent relationship between Harrington and Valier barley was observed in the chemical analysis (Table 2.1), global-source FTIRM (Table 3.7) and synchrotron-source FTIRM (Table 3.9). This nature of this relationship was such that Harrington showed similar or higher amounts of protein (chemical analysis) or Amide I (FTIRM), and higher CHO content than Valier, resulting in a higher starch to protein ratio. Differences in the global-source FTIRM experiment were significant, whereas the

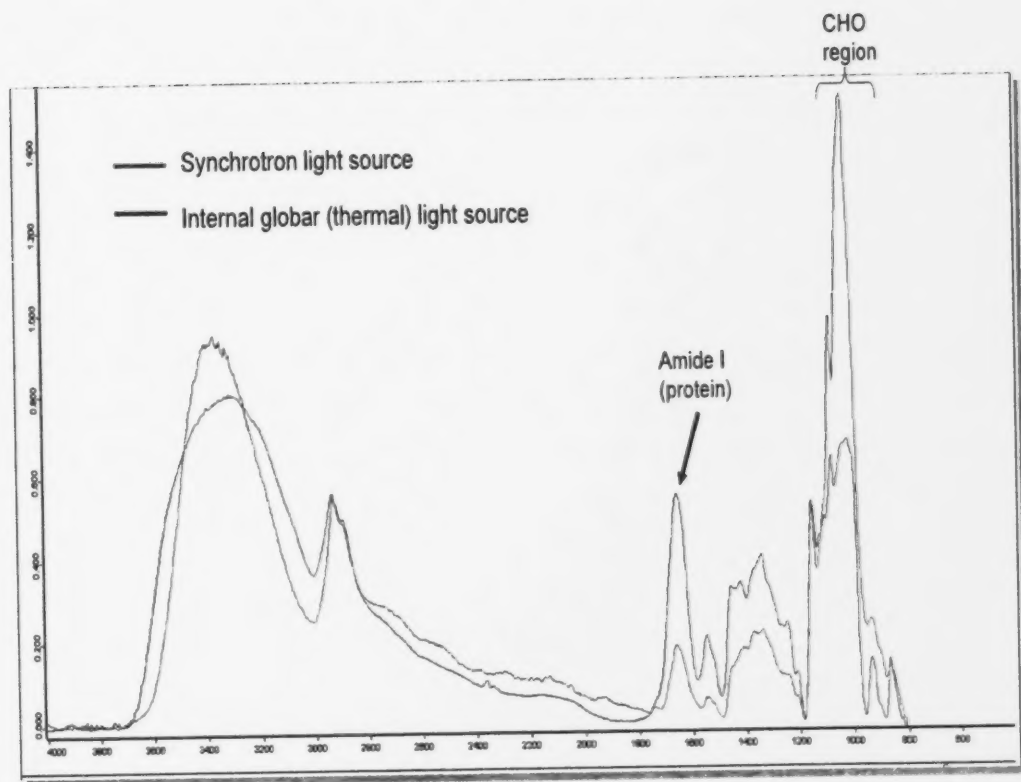


Table 3.9. NSLS synchrotron data: Comparison of least square means of peak area for Amide I and CHO, and ratio of CHO to Amide I for Harrington and Valier barley.

| Variety              | LS Means (Absorbance units) <sup>z</sup> |                  |               |
|----------------------|------------------------------------------|------------------|---------------|
|                      | Amide I <sup>y</sup>                     | CHO <sup>x</sup> | CHO:Amide I   |
| Harrington           | 6.34 (± 3.91)                            | 30.81 (± 13.09)  | 6.68 (± 5.51) |
| Valier               | 6.62 (± 4.25)                            | 31.96 (± 13.54)  | 6.26 (± 3.93) |
| SEM                  | 0.313                                    | 1.039            | 0.361         |
| P-value <sup>w</sup> | 0.5399                                   | 0.4339           | 0.4069        |

Values in the same column with different letters are significantly different ( $P < 0.05$ )

<sup>z</sup> Mean of spot sample spectra ( $n=30$ ) from five different tissue sections for each grain variety. Total  $n$  for each grain = 150.

<sup>y</sup> Amide I peak area measured between 1710 to 1581  $\text{cm}^{-1}$ .

<sup>x</sup> CHO peak area measured between 1066 and 950  $\text{cm}^{-1}$

<sup>w</sup> P-value represents effect of variety

synchrotron data showed similar trends, but was only numerical. Despite this, the evaluation of the synchrotron and global data of Harrington and Valier barley shows them to be consistent and repeatable in the evaluation of chemical components present in barley endosperm tissue. With further experimentation to validate the observations of this study, the quality of information gained from the global light source data may be useful for bulk analysis of plant material to gain a preliminary estimation of the relative content of certain chemical constituents within a grain sample.

### Conclusions

Based on the observations of the FTIRM analysis using both global and synchrotron-source IR light, a lower starch to protein ratio measured using FTIRM techniques seems to correspond with a slower rate of rumen degradation measured by the *in situ* method. This hypothesis, however, appears to hold true only when making comparison between barley varieties. When corn is included in the comparison, neither data obtained from chemical analysis nor FTIRM (both global and synchrotron-source light) support the hypothesis that a lower starch:protein ratio corresponds to slower physical *in situ* degradation characteristics. When estimated using both the chemical analysis and FTIRM analysis, corn had a higher starch:protein ratio (indicating less protein per unit starch), both in the whole kernel and endosperm tissue, than all the barley varieties, yet still exhibited that slowest rate of rumen degradation in the *in situ* trials. This may indicate that it is not necessarily the amount of protein present in the endosperm, but rather the form of the protein which provides the protective qualities alluded to by McAllister et al. (1993). It becomes an issue of protein structure as opposed to protein quantity.

Statistical differences were observed between four barley varieties in the peak area absorption for both CHO and Amide I when using global-source FTIRM. Data from this analysis ranked CDC Bold, CDC Dolly, Harrington and Valier from highest to lowest CHO:Amide I ratio, similar to the ranking of starch:protein ratio estimated by chemical analysis for the four barley varieties, as well as corresponding with the rate and extent of ruminal DM degradation estimated by the *in situ* technique.

No differences in the peak area absorption of CHO or Amide I were apparent between Harrington and Valier barley, nor were there any significant differences observed in the CHO:Amide I ratio. Analysis CDC Bold or CDC Dolly in this study along with Harrington and

Valier would have provided a useful comparison and continuity with the previous global-source FTIR experiment. Based on the observed differences in rate and extent of DM, CP and starch digestibility from the *in situ* trials, coupled with the variation in IR absorption peak areas measured using global-source IR light, a comparison of the IR synchrotron data of CDC Bold and CDC Dolly, as well as additional barley varieties, would certainly be of interest to validate the observations of this experiment and to gain a better understanding of the value of synchrotron IR analysis of cereal grains.

Literature Cited:

References will be included in the final report and are available on request.

**Project 2: Study of flaxseed inherent structures affected by heat processing in relation to utilization and availability in ruminants.**

Research was carried out by M.Sc. graduate student Mr. Kevin Doiron under the supervision of Dr. Peiqiang Yu. Mr. Doiron's work centered on the effects of feed processing on protein secondary structure and nutrient availability in the digestive tract. Beam time in July 2006 was obtained for SR-FTIR evaluation of flaxseed tissues at NSLS in Brookhaven, NY.

**ABSTRACT:**

**List of abbreviations**

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|         |                                                                                                                                                                                                                                             |
|---------|---------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|
| ADIP:   | Acid detergent insoluble protein                                                                                                                                                                                                            |
| CLA:    | Hierarchical cluster analysis                                                                                                                                                                                                               |
| CNCPS:  | Cornell Net Carbohydrate and Protein System                                                                                                                                                                                                 |
| CP:     | Crude protein                                                                                                                                                                                                                               |
| IR:     | Infrared                                                                                                                                                                                                                                    |
| NDIP:   | Neutral detergent insoluble protein                                                                                                                                                                                                         |
| DRIFT:  | Diffuse reflectance infrared Fourier transform                                                                                                                                                                                              |
| NPN:    | Non-protein nitrogen                                                                                                                                                                                                                        |
| PA:     | Protein sub-fraction A described in CNCPS; Fraction of CP that is instantaneously solubilized at time zero                                                                                                                                  |
| PB1:    | Protein sub-fraction B1 described in CNCPS; Fraction of CP that is soluble in borate-phosphate buffer and precipitated with trichloroacetic acid                                                                                            |
| PB2:    | Protein sub-fraction B2 described in CNCPS                                                                                                                                                                                                  |
| PB3:    | Protein sub-fraction B3 described in CNCPS                                                                                                                                                                                                  |
| PC:     | Protein sub-fraction C described in CNCPS; Fraction of CP recovered with ADF and is considered to be undegradable. It contained proteins associated with lignin and tannins and heat-damaged proteins such as the Mailard reaction products |
| PCA:    | Principal components analysis                                                                                                                                                                                                               |
| SCP:    | Soluble crude protein                                                                                                                                                                                                                       |
| SFTIRM: | Synchrotron radiation-based Fourier Transform Mid-IR Microspectroscopy                                                                                                                                                                      |

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## Introduction

Current methods of food/feed evaluation, particularly for protein quality, are typically slow and cumbersome processes that require the destruction of a sample for any given measurement during "wet" chemical analysis. The information gleaned from such procedures is also not totally in context; that is to say some pertinent information is lost or not knowable by the process of taking the measurements. Part of the reason for this is that these standard methods destroy any spatial information as well as the distribution of those elements of interest (Budevskia 2002). These elements may be linked to degradability and digestibility in human/animals.

Vibrational spectroscopy or mid-Infrared spectroscopy has the capacity to overcome some of the issues with modern food/feed characterization methods. The results from mid-IR spectroscopy are complex in nature the information contained within each spectra is related not only to the chemical bonds in each sample but interrelations between these chemical bonds (Novikov et al. 1998). So rather than look at the components of interest separate from one another, we can look at everything in context.

When a synchrotron (which is million times brighter than sunlight) is used as the source of infrared light it is known as Synchrotron-based FTIR Microspectroscopy (SFTIRM). This coupling permits an even greater amount of information to be gathered from a sample of interest at both the cellular and molecular levels. The qualities of synchrotron light, including brightness and source size, permit IR spectroscopic measurements to be made on a sub-cellular scale thus allowing us to link quantity, interrelationships, and localization of compounds of interest all in a single experiment (Wetzel and Levine, 2001). This mid-IR technique permits a greater understanding of food/feed structures at the cellular level (Yu 2004c), and with further work will allow a more complete characterization of those food/feed traits related to quality.

One feature of protein that can be quantified with mid-IR methods is its secondary structure which has some links to protein digestibility and degradability for example (Yu et al. 2004a). This is because digestion is a complex process where any kind of steric issues at the molecular level, such as those presented by the different secondary structures, will ultimately affect the access enzymes will have to the food/feed constituents. Heating has also been demonstrated to alter the relative amounts of each secondary structure thus having possible connections to alterations in degradability (Yu 2005).

The objective of this study was to use both DRIFT and SFTIRM techniques as a means of detecting heat induced changes to food/feed protein. Using multivariate molecular spectral analysis techniques as well as measuring  $\alpha$ -helix and  $\beta$ -sheet ratios should allow us to identify molecular differences between different autoclaved flaxseed treatments. The goal was to attempt to understand and identify spectroscopic signatures that are associated with changes in degradability. The hypothesis of this study was that autoclaving would induce spectroscopic changes to the flaxseed spectra that we can characterize with DRIFT and SFTIRM and may have a link to differences in degradability and digestibility in animal/human.

## **MATERIALS AND METHODS**

### ***Flaxseed heating and processing***

Flaxseed (*Linum usitatissimum*) (cv. Vimy) grown near Moose Jaw, Saskatchewan during the 2005 growing year was provided by Shamrock Seeds Ltd. (Saskatoon, Saskatchewan). Flaxseed were heated by autoclave (Amsco Eagle SG-3031, STERIS Corporation) in an autoclave trays at temperature 100°C for 20, 40 and 60 min as treatment 1, 2 and 3, respectively. Control treatments were kept raw.

### ***Chemical Analysis***

All samples for chemical analysis were ground through a 1 mm screen. Crude protein (CP, AOAC official method 984.13) contents were analyzed according to the procedure of the AOAC (1990). The acid (ADIN) and neutral detergent insoluble N (NDIN) values were determined according to the procedures of Licitra et al. (1996). The non-protein N (NPN) content was analyzed by precipitating of true protein with tungstic acid and calculated as the difference between total N and the N content of the residue after filtration (Licitra et al. 1996). Total soluble crude protein (SCP) was determined by incubating the sample with bicarbonate-phosphate buffer and filtering through Whatman #54 filter paper (Roe et al. 1990). The detailed chemical characteristics are present in Table 1.

### ***Partitioning Protein and Carbohydrate Fractions***

The crude protein (CP) and carbohydrate (CHO) subfractions were partitioned according to the Cornell Net Carbohydrate and Protein System (CNCPS) (Sniffen et al., 1992; Chalupa and Sniffen, 1994). The characterization of the CP fractions as applied in this system is as follows: fraction PA is non-protein N (NPN), fraction PB is true protein (TP), and fraction PC is unavailable protein. Fraction PB is further divided into three fractions (PB1, PB2, and PB3) that are believed to have different rates of degradation in the rumen (Sniffen et al., 1992). Buffer-insoluble protein minus fraction PB3 is used to estimate fraction PB2. Fraction PB2 is insoluble in buffer but soluble in neutral detergent, while fraction PB3 is insoluble in both buffer and neutral detergent, but is soluble in acid detergent solution. Fraction PB2 is fermented in the rumen at a lower rate than the buffer-soluble fraction, and some PB2 fraction escapes to the lower gut. Fraction PB3 is believed to be more slowly degraded in the rumen than fractions PB1 and PB2 because of its association with the plant cell walls; a large proportion of PB3 is thus believed to escape the rumen. Fraction PC is ADIN, which is highly resistant to breakdown by microbial and mammalian enzymes, and it is assumed to be unavailable to the animal.

The relative rumen degradation rates of the five protein fractions have been described by Sniffen et al. (1992) as follows: fractions PA is rapidly degradable with an assumed degradation rate to be infinity, fraction PB1 is rapidly degradable with a degradation rate of 120-400 %/h, fraction PB2 is intermediately degradable and has a degradation rate of 3-16%/h, fraction PB3 is slowly degradable with a degradation rate of 0.06-0.55%/h, and fraction PC is unavailable and considered to be undegradable. The detailed biochemical characteristics associated with degradation kinetics in the rumen are present in Table 1.

### ***Diffuse Reflectance Fourier Transformed Infrared Spectroscopy (DRIFT)***

Cooled flaxseed samples were ground by coffee grinder (Braun KSM 2), chilled again and re-ground. Grinding was done for 3 min each time. The cooling was done so as to prevent the samples from forming dough and from getting too warm, thus creating artefacts in the spectra. The coffee grinder was selected to minimize the effects of grinding on the flaxseed spectra ensuring the physical cracking and breaking of the sample yet maintaining as much of the smaller plant seed structures and molecular bonds as possible. Samples of the ground flaxseed were then mixed with KBr in a ratio of 4 parts flaxseed to 1 part KBr in a 2 mL centrifuge tube

and mixed by vortex for several min. DRIFT was performed using a Bio-Rad FTS-40 with a ceramic IR source and MCT detector (Bio-Rad laboratories, Hercules, California, USA). Data was collected using Win-IR software. Spectra were generated from the mid-IR (4000-800  $\text{cm}^{-1}$ ) portion of the electromagnetic spectrum with 256 co-added scans and a spectral resolution of 4  $\text{cm}^{-1}$ . Spectral analysis was done with OMNIC 6.0 (Spectra Tech) software.

### ***Synchrotron SFTIRM window preparation***

Randomly selected flaxseeds from treatment samples were sectioned to a thickness of 6  $\mu\text{m}$  by microtome (Microm 330 (Microm Laborgerate GmbH, Sandhausen, Germany) at the Western College of Veterinary Medicine, University of Saskatchewan, Canada. The detailed preparation procedure was reported in Walker (2007). Sample sections were subsequently transferred to  $\text{BaF}_2$  discs (Spectral Systems, NY; part number 915-3015). Each  $\text{BaF}_2$  disc had 3 separate sections from 3 separate flaxseeds placed on it. Fourteen seeds per treatment were used from each treatment to generate 10 spectra of randomly selected spots within the endosperm of each seed. More spectra were gathered when a collected spectrum contained excessive noise. Figure 1 shows the photomicrograph of cross-section of flaxseed (cv. vimy) seed showing the intrinsic structure – the pericarp (outside of seed coat), seed coat, aleurone and endosperm (magnification: 10 $\times$ 20). Picture was taken using Olympus Compound Microscopy with digital camera Model BX41TF, Olympus Optical Co. Ltd.

### ***Synchrotron FTIR microspectroscopy(SFTIRM)***

Synchrotron based FTIR microspectroscopy was performed on beam line U2B at the National Synchrotron Light Source located in Brookhaven National Laboratory (NSLS, BNL, Department of Energy, Upton, New York, USA). Collimated light from the synchrotron was directed to an FTIR spectrometer (Nicolet Magna 860) with a KBr beam splitter and a nitrogen cooled mercury cadmium telluride (MCT-A) detector through a port on the spectrometer. The spectrometer was interfaced with a Nic PLAN IR microscope using a single image plane mask set to give an aperture of 10 $\mu\text{m}$  x 10 $\mu\text{m}$ . Spectra were generated in transmission mode with the mid-IR (4000-800  $\text{cm}^{-1}$ ) portion of the electromagnetic spectrum by 128 co-added scans with a spectral resolution of 4  $\text{cm}^{-1}$ . Spectral analysis was done with OMNIC 6.0 (Thermo-Nicolet, Madison, WI, USA) software. Spectra were generated from randomly selected regions of the endosperm of the flaxseed sections.

### ***Alpha helix to beta sheet ratio measurement***

Protein secondary structures were estimated using the amide I functional group band located in the region of ca.  $1700\text{ cm}^{-1}$  –  $1610\text{ cm}^{-1}$ . Two different methods were used to estimate relative protein secondary structure concentrations for DRIFT spectra. In the first method the spectra were first converted by Fourier self-deconvolution with OMNIC 6.0 (Spectra tech., USA) to identify the component peaks and frequencies that comprise the amide I band. The area of the component bands representing  $\alpha$ -helices and  $\beta$ -sheets in the FSD spectra were then calculated using Origin data analysis software (Origin Lab Corporation, Northampton, MA, USA) with both the Gaussian and Lorentzian multi component peak modeling methods. The area under the peak at ca.  $1658\text{ cm}^{-1}$  was taken to represent amide I bonds in the  $\alpha$ -helical conformation while the area under the peak at ca.  $1630\text{ cm}^{-1}$  was the amide I bonds in the  $\beta$ -sheet conformation. The second method was used for both DRIFT and SFTIRM spectra and involved using the FSD spectra generated with OMNIC 6.0 to identify the region of the absorption spectra representing the respective secondary structures. OMNIC 6.0 was then used to calculate the area in the absorption spectra representing the  $\alpha$ -helices and  $\beta$ -sheets.

### ***Multivariate analysis of protein spectra***

CLA and PCA multivariate statistical methods were used to compare the DRIFT spectra of the different treatments to the control samples to determine if there were some underlying structural differences in the spectral information that would permit the identification of spectra belonging to different treatment groups. First the absorbance spectra were baseline corrected using the automatic baseline correction function of OMNIC 6.0. The spectra were then analyzed together using Statistica 6.0 software (Statsoft, Tulsa, OK, USA). For CLA and PCA the spectral regions of  $2000 - 800\text{ cm}^{-1}$ , containing most of the mid-IR spectrum, and  $1715 - 1485\text{ cm}^{-1}$ , containing the amide I and II regions, was used. CLA results were presented as dendograms while PCA results were plotted based on the two highest factor scores and plotted as a function of those scores. In each comparison the eigenvector for factor 1 was plotted against that for factor 2 which accounted for over 99% of the variability in the data.

### Statistical analysis

Statistical analyses of protein secondary structure ratios were performed using the MIXED procedure of SAS (version 9.1). DRIFT spectra were analyzed in a completely randomized design using the model:  $Y = \text{mean} + \text{treatment} + \text{error}$ .

SFTIRM spectra were analyzed in a nested experimental design with the model:  $Y = \text{mean} + \text{treatment} + \text{seed (nested within treatment)} + \text{error}$ . Significance was taken as  $P < 0.05$ .

### RESULTS AND DISCUSSION

Results from the protein secondary structure analysis based on the DRIFT spectra are presented in Table 2. It is important to note that these ratios are based on relative values of the secondary structures present. Using an  $\alpha$ -helix to  $\beta$ -sheet ratio to assess changes to protein requires many considerations. From IR spectroscopy, it may be not possible to get exact values for each secondary structure, but if all sample spectra are treated in the same manner, we can compare the results from treatment to another (Yu 2006a). In addition to this, because of different methodologies used between researchers, results from one study can be different from another considering the same biological tissue. This arises from the complexity of the information contained within the mid-IR spectrum. Selecting different regions of the spectrum and different sizes for those regions around the amide I and II bands along with different baselines are all factors that would influence the resulting  $\alpha$ -helix to  $\beta$ -sheet ratio (Yu 2006a). There exist technical issues that affect protein secondary structure analysis as well. The amide I band carbonyl group has different sensitivities to absorption depending on secondary structure, the number of bands it contains varies and is usually not known for certain, and finally the bands can be varied in shape (Yu 2006a). Even for relative determinations of the  $\alpha$ -helix to  $\beta$ -sheet ratio we must be aware of these facts. Despite these issues the  $\alpha$ -helix to  $\beta$ -sheet ratio can be a useful way of exploring differences in protein structure that exist between the treatments, varieties, etc.

What is clear in all three different ratio measurements is that the ratio of  $\alpha$ -helices to  $\beta$ -sheets decreased significantly upon heating ( $P < 0.05$ ). This change is in accordance with those published by Yu et al. (2005) using golden flaxseed and the SFTIRM technique with roasting as heating method. Of the three methods used to estimate the relative  $\alpha$ -helix to  $\beta$ -sheet ratios only the area under peak method was able to discern any difference between the treatments. These

changes in the protein secondary structure ratio are likely due to more  $\alpha$ -helices being denatured or destroyed during the autoclaving process than  $\beta$ -sheets. These estimates however seem to disagree with results published by Madhusudhan and Singh (1985a, b) who reported that the major proteins of flaxseed, albumin and globulin, consist predominantly of  $\beta$ -sheet secondary structures as determined by the circular dichroism (CD) technique using purified protein samples. It is possible, however, that the remaining 14% of protein in flaxseed outside these two classes have extremely high concentrations of  $\alpha$ -helices relative to other secondary structure types. It should also be noted that there is some degree of variability in terms of oil and protein content for varieties, growing conditions are also known to affect content as well, which means the differences in the reported values could be explained in this manner.

In Table 3 the results of the same analysis are shown for S-FTIR data. The  $\alpha$ -helix to  $\beta$ -sheet ratio increased upon heating which differed from the DRIFT results. The control sample and the 20 min autoclave treatment were not significantly different from one another as was the case for the 40 min and 60 min treatment samples. One possibility for the difference in the effect autoclaving has on the  $\alpha$ -helix to  $\beta$ -sheet ratio between the two mid-IR spectroscopic methods is that DRIFT spectra were from whole flaxseeds which includes the seed coat, pericarp, aleurone layer and endosperm (Figure 1). The aleurone layer is rich in protein and its composition may be different from that of the endosperm. The synchrotron SFTIRM spectra on the other hand were generated solely from the endosperm region of the flaxseed. Furthermore, it is possible that the individual regions may have different sensitivities to heating which may cause different changes to secondary structure ratios to different flaxseed tissues. Further study is required to understand the effect of heating on different tissues within flaxseed.

Cluster analysis for the ca. 2000-800  $\text{cm}^{-1}$  spectral (Fingerprint) region was carried out based on DRIFT spectra (all figures are given in Appendix A). Other than a single spectrum from the 20 min autoclaved treatment 1 the raw spectra find themselves in their own cluster. The remaining clusters all contain combinations of spectra from the other treatments. This implies that DRIFT spectroscopy combined with cluster analysis can nearly distinguish autoclaved flaxseed samples from raw samples and the benefit of cluster analysis itself is that no other prior knowledge of the sample other than a mid-IR spectrum is required to make such a distinction. The cluster analysis comparisons show similar results to those displayed in Figure 3 (Appendix A). Figure 3a shows a comparison between the control spectra and treatment 1 spectra. Here a

single spectrum from treatment 1 is clustered among the control samples. In Figure 3b the treatment 2 spectra and the control spectra are all found within distinct clusters although the dendrogram shows that some of the treatment 2 spectra are more closely related to the control spectra. Figure 3c shows a very clear separation between the control spectra and treatment 3 spectra with all spectra falling within their respective clusters. Figure 3d shows a distinct cluster for the treatment 2 spectra, however, not all the spectra were from treatment 2 were similar enough to be assigned to this group. The remaining CLA comparisons (Figures 3e,f) failed to demonstrate any distinct groupings among the treatments.

Figure 4 (Appendix A) shows the results of the principal components analysis conducted using DRIFT spectra. The results show a similar grouping of the raw flaxseed spectra with a single spectrum from the 20 min autoclaved treatment 1. The agreement of both PCA and cluster analyses suggests that there is some similar structure in the data, in this case the baseline corrected spectra, which distinguishes almost completely the raw sample spectra from those spectra of autoclaved samples. The plot was derived from values for the first two principal components generated by the analysis which explain 95.88% and 3.42% of the variability in the spectra. Factor 1 in this case can almost discriminate entirely between the raw and treated flaxseed samples and represents the vast majority of the variability present. As in the case for cluster analysis Yu et al. (2007) showed that PCA could also distinguish between plant structures. We can conclude that not only can we use FTIR based experimental methods with little modification to the spectra to identify treated samples but also to identify which part of the plant the spectra were derived.

Further PCA spectral comparisons are shown in Figure 5 (Appendix A). Figure 5a shows the results from PCA between the control and the treatment 1 spectra. Similar to the CLA results Figure 5a shows that a single spectrum from the first treatment is indistinguishable from the control samples. In Figures 5b, c, the control spectra are found in their own group with treatment spectra spread out more but not mixed up in the areas occupied by control spectra. This shows a bit of distinction from the CLA results for the treatment 2 spectra where there was still some linkage to the control samples. The remaining spectral comparisons (Figures 5d, e, f) by PCA were not able to show any clear group of the spectra on the factor plane.

CLA and PCA were subsequently conducted on a more defined region of the spectrum, in this case the spectrum from ca. 1715-1485  $\text{cm}^{-1}$  which covered both the prominent amide I and

II bands. Figure 6 (Appendix A) shows the CLA results from this region of the spectrum for all treatment spectra. It shows a similar cluster pattern to that in Figure 2 with the exception that in it there is a distinct cluster for the treatment 2 spectra although not all of the treatment 2 spectra are contained within it. It also shows that the control samples are found in their own group with a single spectrum from treatment 1. Figure 7b shows CLA results for the treatment 2 to control spectral comparison. This analysis shows an improvement of the clustering ability when we narrowed the spectral window, in this case both the control spectra and the treatment 2 spectra are found in their own unique clusters as opposed to when a much larger portion of the spectrum was used. This indicates that the protein structures were different between raw flaxseed and that from treatment 2 this is similar to the results between raw flaxseed and that from treatment 3. This improvement in clustering is also seen in Figure 7f where we find clusters for treatments 2 and 3 although there still remains some mixing of the spectra in the remaining clusters.

The PCA analysis shows similar improvements upon selecting a smaller spectral window. In Figure 8 (Appendix A) where all spectra are included in a PCA there is no longer a spectrum from the treatment 1 group present amongst the control sample spectra. Upon further examination of the comparisons between the treatments shown in Figure 9a, b, c (Appendix A) we can see that autoclaved treatments are completely separated from the raw spectra. The separation indicated that treatment spectra differ from the raw spectra and suggested that there was a molecular structure change in the protein based on the amide I and amide II regions.

While it appears as though some more work is required to establish how we can use protein secondary structures to forecast degradability of a feed after a given treatment, these changes are the result of autoclave treatment and cooking in general (Arnoldie 2001) which will influence degradability. It may be that the two primary secondary structures need to be supplemented with information about other secondary structures such as random coils to use this measure more accurately in predicting degradability. It is also evident that there needs to be a standardized and well controlled procedure for sample preparation as demonstrated by the difference in the relative ratios of  $\alpha$ -helices to  $\beta$ -sheets as measured by multiple techniques using both DRIFT and S-FTIR.

It could be erroneous to rely solely on protein secondary structure ratios to predict digestibility. It has been shown that feathers are poorly degradable protein sources such and are rich in beta sheets (Yu et al. 2004b). This, however, should not preclude feeds that are low in  $\alpha$ -

helices and high in  $\beta$ -sheets from being rapidly degraded in the rumen as the  $\beta$ -keratins that make up the feather have other molecular properties rendering them insoluble and poorly degraded such as a high concentration of disulfide bonds. Hair is made up of  $\alpha$ -keratin which is high in  $\alpha$ -helices and cytokine bonds. Hair, like feathers, has been shown to be poorly degraded as well (Thomas and Beeson 1977). Further investigation into the digestibility and molecular chemistry of these two poorly degraded proteins may provide some insight into how secondary structure influences degradability.

Comparisons of protein secondary structure ratios for other feed sources may only be applicable when dealing with a single variety within the same genus and species. Even when considering the same variety, growing conditions and even topography have the capacity to alter protein content of plants (Kravchenko and Bullock, 2002). Changes to protein quality will be reflected by different amounts of the various storage proteins specific to each plant (Paek et al. 1997) which means that the initial  $\alpha$ -helix to  $\beta$ -sheet ratio could be altered by these conditions and after processing may show different shifts to secondary structures as a result.

The results from the multivariate statistical methods used in this study show that by focusing in on specific regions of the spectrum we can enhance our ability to segregate the spectra based on treatment conditions. This is likely a result of eliminating some extraneous data that bogs down the analysis or, in the case of autoclaving, data for peaks that are minimally influenced by the treatment. In IR spectra a large region, found between ca. 2500-2000  $\text{cm}^{-1}$ , is usually devoid of any peaks of interest so removing data contained in regions such as these from the analysis is justifiable. Selecting windows or regions of the spectra will also allow us to determine which regions, representing certain molecules, are more or less affected by a given treatment.

For multivariate analyses very little modification of the spectra and thus very little time is required for this analysis. The implications of this are that with a pool of spectra we would only need to add an unknown spectrum of interest to the cluster analysis procedure to discover if the sample has been autoclaved or not, provided all other things remain equal such as sample preparation for example. The 20 min autoclave procedure is a relatively mild treatment so the ability to make this distinction is indicative of the potential of this methodology. These results are further validated by those reported by Yu et al. (2007) where using S-FTIR spectra and also

without prior modifications of the spectra cluster analysis was able to distinguish between those spectra from various wheat structures such as endosperm and the aleurone layer.

## **CONCLUSIONS AND IMPLICATIONS**

The multivariate spectral analyses employed in this experiment showed that, in terms of classification, they can provide a clear idea of degradability through association to the treatment conditions. Linking these results to degradability itself rather than treatment conditions might prove to be a more effective way of using these statistical tools for this sort comparison. Similar FTIR methodologies have been used to identify bacteria in the past classifying different strains of the same *genus* (Maquelin et al 2002) so there is a vast potential untapped for FTIR whether DRIFT or S-FTIR based spectroscopies are employed in the food/feed industry. Further investigation into the influence of protein secondary structure in relation to degradability and digestibility is required.

**Table 1.** Biochemical Characterization of flaxseed (cv. Vimy): comparison of the raw (control) vs. the treated at various times and temperatures

| vs. the treated at various times and temperatures                |                   |                   |                   |                   |      |
|------------------------------------------------------------------|-------------------|-------------------|-------------------|-------------------|------|
|                                                                  |                   | Treatment         |                   |                   |      |
|                                                                  | Control           | 1                 | 2                 | 3                 | SEM  |
| Protein profiling                                                |                   |                   |                   |                   |      |
| CP (%DM)                                                         | 23.9              | 23.7              | 23.8              | 23.8              | 0.13 |
| SCP (%DM)                                                        | 12.4 <sup>a</sup> | 8.5 <sup>b</sup>  | 5.4 <sup>c</sup>  | 4.5 <sup>c</sup>  | 0.32 |
| NPN (%DM)                                                        | 1.4 <sup>b</sup>  | 1.3 <sup>b</sup>  | 1.5 <sup>b</sup>  | 2.0 <sup>a</sup>  | 0.10 |
| NDIP (%DM)                                                       | 3.8 <sup>b</sup>  | 4.1 <sup>b</sup>  | 4.4 <sup>b</sup>  | 5.7 <sup>a</sup>  | 0.13 |
| ADIP (%DM)                                                       | 0.5 <sup>c</sup>  | 0.6 <sup>bc</sup> | 0.7 <sup>ab</sup> | 0.8 <sup>a</sup>  | 0.04 |
| Protein subfractions associated with degradation characteristics |                   |                   |                   |                   |      |
| PA (%CP)                                                         | 6.0 <sup>b</sup>  | 5.3 <sup>b</sup>  | 6.4 <sup>b</sup>  | 8.5 <sup>a</sup>  | 0.41 |
| PB1 (%CP)                                                        | 45.9 <sup>a</sup> | 30.7 <sup>b</sup> | 16.5 <sup>c</sup> | 10.5 <sup>d</sup> | 0.99 |
| PB2 (%CP)                                                        | 43.0 <sup>c</sup> | 58.3 <sup>b</sup> | 71.0 <sup>a</sup> | 73.3 <sup>a</sup> | 1.09 |
| PB3 (%CP)                                                        | 3.3 <sup>b</sup>  | 3.5 <sup>b</sup>  | 3.7 <sup>b</sup>  | 4.9 <sup>a</sup>  | 0.15 |
| PC (%CP)                                                         | 1.9 <sup>c</sup>  | 2.2 <sup>bc</sup> | 2.5 <sup>ab</sup> | 2.8 <sup>a</sup>  | 0.11 |

<sup>a, b, c, d</sup> Means with same superscripts in the same row are not significantly different ( $P>0.05$ ) LSD method; SEM = Standard error of mean. Control=raw; 1= 20min@120°C; 2=40min@120°C; 3=60min@120°C

**Table 2.**  $\alpha$ -helix to  $\beta$ -sheet ratios from DRIFT spectra of the autoclaved vimy flaxseed

|                                          | Control           | Treatment         |                   |                    | SEM   |
|------------------------------------------|-------------------|-------------------|-------------------|--------------------|-------|
|                                          |                   | 1                 | 2                 | 3                  |       |
| 1. DRIFT method (Area under peak method) | 2.18 <sup>a</sup> | 1.60 <sup>b</sup> | 1.27 <sup>c</sup> | 1.37 <sup>c</sup>  | 0.011 |
| 2. Gaussian Modeling                     | 2.08 <sup>a</sup> | 1.06 <sup>b</sup> | 0.99 <sup>b</sup> | 1.23 <sup>b</sup>  | 0.072 |
| 3. Lorentzian Modeling                   | 2.27 <sup>a</sup> | 1.07 <sup>b</sup> | 1.10 <sup>b</sup> | 1.72 <sup>ab</sup> | 0.071 |

<sup>a, b, c</sup> Means with same superscripts in the same row are not significantly different ( $P>0.05$ ). Means separated using the LSD method. Control=raw; 1= 20min@120°C; 2=40min@120°C; 3=60min@120°C

**Table 3.**  $\alpha$ -helix to  $\beta$ -sheet ratios in the endosperm area of the autoclaved flaxseed as determined using the area under peak method and synchrotron SFTIRM data

|                                        | Control           | Treatment         |                   |                   | SEM   |
|----------------------------------------|-------------------|-------------------|-------------------|-------------------|-------|
|                                        |                   | 1                 | 2                 | 3                 |       |
| SFTIRM method (Area under Peak method) | 0.86 <sup>b</sup> | 0.88 <sup>b</sup> | 1.03 <sup>a</sup> | 1.03 <sup>a</sup> | 0.003 |

<sup>a, b</sup> Means with same superscripts in the same row are not significantly different ( $P>0.05$ ). Means separated using the LSD method. Control=raw; 1= 20min@120°C; 2=40min@120°C; 3=60min@120°C

### **Project 3: Phosphorus Speciation of Dried distiller's Grains with Solubles**

**Project Lead: Dr. Colleen Christensen**

#### **Introduction**

Profitability of ethanol production is linked to a number of factors including access to a stable supply of quality feedstock, production costs, market value of ethanol, and the value of ethanol co-products for sale. Phytate (inositol hexakisphosphate, IP6), is the main storage form of phosphorus in plant tissue. Phytate is not biologically available to livestock. If phytate is not modified by enzyme treatment, phytate will pass through the animal and eventually the land around intensive livestock areas can have environmental problems associated with an imbalance of phosphorus in the soil. The total phosphorus content of dried distillers grains, the main ethanol manufacturing co-product, contains ~ 1.0 – 1.2% phosphorus. This content is high enough that a current University of Saskatchewan research trial pertaining to the upper limits of inclusion of distillers grains in animal diets contains an associated manure management research component that is monitoring nitrogen and phosphorus levels. This project will investigate the forms of phosphorus in wheat flour, wheat based distillers grains as well as in relevant phosphorus reference compounds in order to understand whether the bioethanol process may increase the bioavailability of phosphorus for livestock nutrition.

#### **Scientific Merit**

Phytate (inositol hexakisphosphate, IP6) is the main storage form of phosphorus in plant tissue. Phytate combines with cations in the digestive tract of animals to form insoluble complexes (Reale et al., 2007). Once these complexes are formed, the phosphorus constituent cannot be utilised by animals unless phytate is hydrolysed by phytase. Bacteria fermentation of cereals has been shown to reduce the phytate content of cereals. Widyaratne and Zijlstra (2007) determined the total phosphorus content and forms of phosphorus in distillers grains utilizing high performance liquid chromatography (HPLC). The PGM beamline is a beamline with soft X-rays

that is in the appropriate range for evaluating the L-edge absorption spectra for phosphorus. The L-edge absorption region would be able to provide most of the details about the chemical speciation of phosphorus.

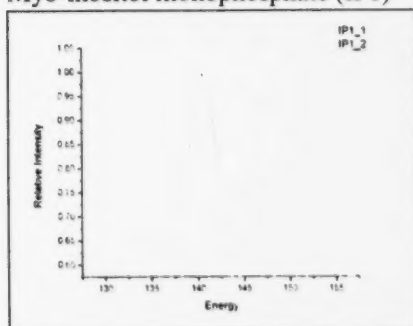
### Experimental Procedure

Wheat grain and dried distillers grains with solubles (DDGS) samples obtained from different local bioethanol facilities were ground to a 0.5 mm mesh size. At the CLS, DDGS was placed on double sided tape on a VLS-PGM sample holder. The samples will be placed in the beamline chamber and the spectra obtained and analysed using origin lab software. Reference samples (myo-inositol monophosphate (IP1), myo-inositol diphosphate (IP2), isomers of myo-inositol triphosphate (IP3), isomers of myo-inositol tetraphosphate (IP4) and myo-inositol pentaphosphate (IP5) and inositol hexakisphosphate (IP6 or phytate) were tested and compared to the flour and DDGS samples.

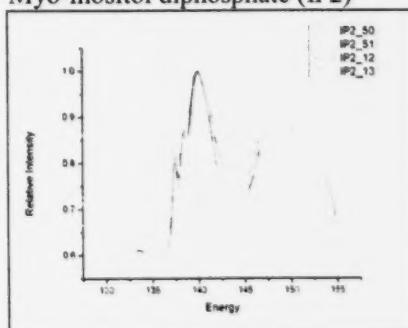
### Results

The following results are phosphorus L-edge spectra of the analytical grade reference compounds. The graphs represent raw data which has not been processed.

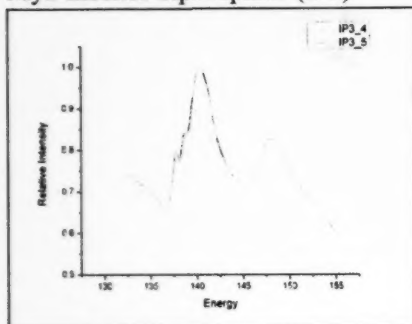
Myo-inositol monophosphate (IP1)



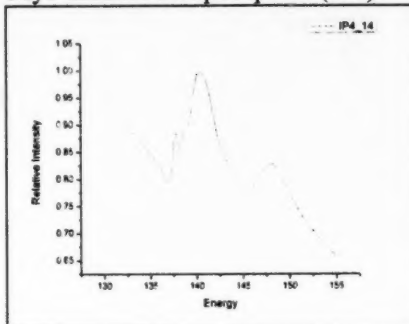
Myo-inositol diphosphate (IP2)



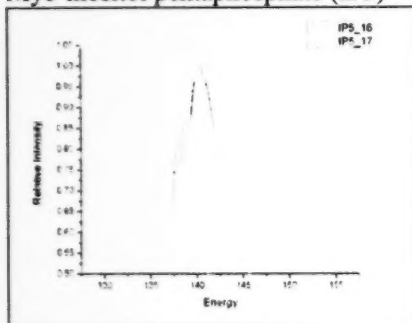
Myo-inositol triphosphate (IP3)



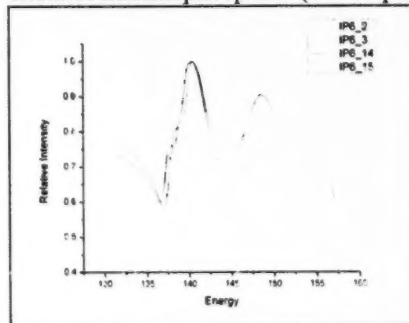
Myo-inositol tetraphosphate (IP4)



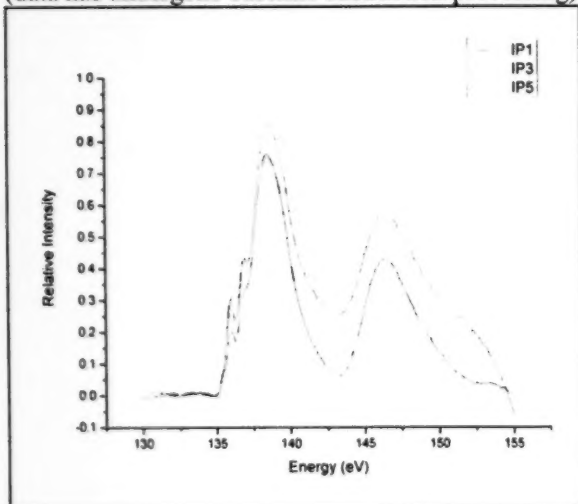
Myo-inositol pentaphosphate (IP5)



Inositol hexakisphosphate (IP6 or phytate)



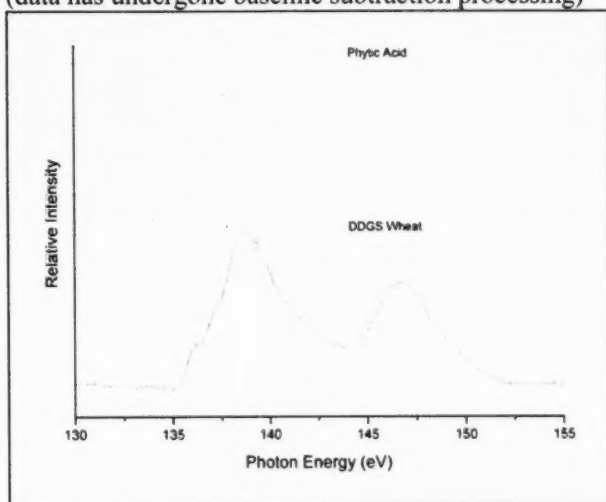
Summary graph of three reference compounds  
(data has undergone baseline subtraction processing):



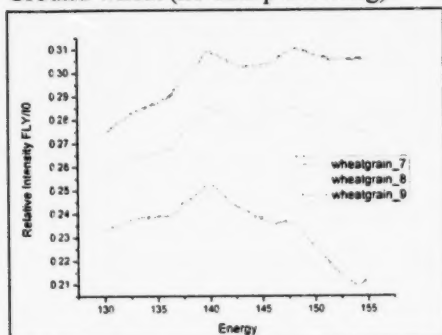
Comparison of analytical grade reference compounds indicates that addition of phosphate groups results in a change in the triplex peak structure of the near-edge spectra, similar to that of Bancroft et al. (1997). With an increase in phosphate groups the three peaks increase in intensity relative to each other.

The following graphs represent the samples from bioethanol facilities or ground grain which potentially contain multiple phosphorus species. The graphs represent partially processed or raw data which has not been processed.

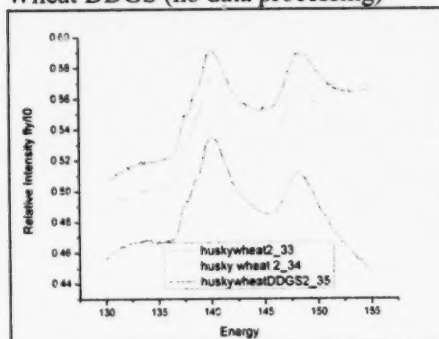
Comparison of analytical grade phytic acid to wheat DDGS  
(data has undergone baseline subtraction processing)



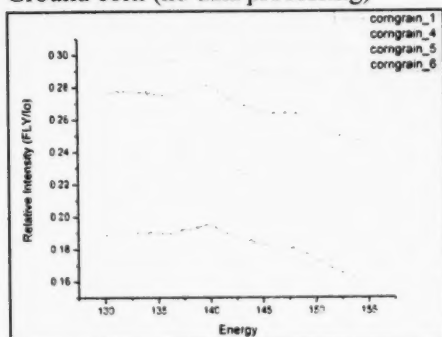
Ground wheat (no data processing)



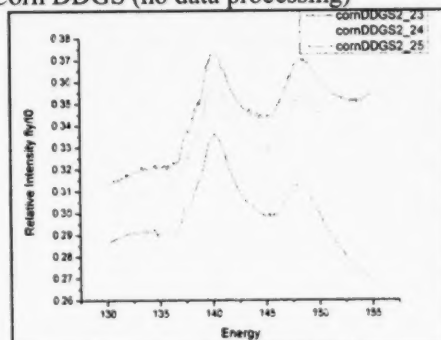
Wheat DDGS (no data processing)



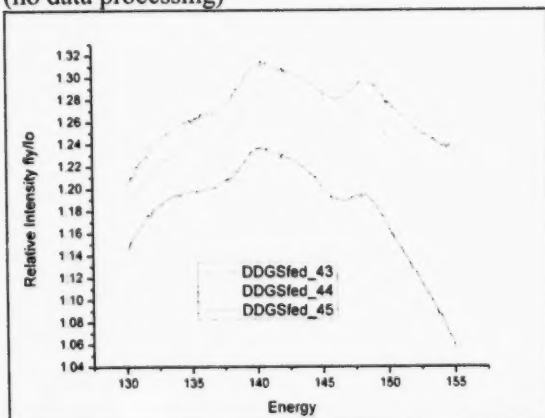
Ground corn (no data processing)



Corn DDGS (no data processing)



Manure from beef cattle fed wheat DDGS (no data processing)



The data graphed above is primarily the raw spectra captured from the PGM beamline without data processing. Although noise exists in each spectra, the levels of phosphorus measured in each sample would be sufficient to undergo data processing. However, significant differences in baselines were obtained throughout the synchrotron run. The PGM beamline operates under a significant vacuum. Differences in the baselines can be created for various reasons including: not allowing for sufficient time for the vacuum to re-establish between sample changes and volatiles released from the sample interfering with the vacuum. Issues with baseline fluctuations were not anticipated prior to the initiation of this experiment. This experiment was planned with the input of the PGM beamline scientist, however the majority of samples analyzed using this method are not organically based. The behaviour of biological samples in a synchrotron beamline under vacuum has not been well characterized.

The data from these experiments is currently being evaluated through several data processing procedures to determine if a common baseline subtraction method can be created so that data from the samples can be compared qualitatively or quantitatively.

It is anticipated that this data will eventually be published, however the data may only be to the quality of a technical note: such as the CLS annual report, rather than being published in a peer review journal.

Although there are difficulties in processing of this phosphorus data, the results to date are promising enough that a future experiment and CLS PGM beam time has been obtained in collaboration with Dr. Derek Peak, UofS Soil Science. The idea for this future experiment, as well as data processing, was established through the collaborations established within the Feed Opportunities from the Biofuels Industries and existing collaborations between Derek Peak and Jeff Schaoneau. Additional reference compounds have been purchased. Samples will be analyzed that were created through the MSc thesis work of Ms. Lee-Anne Walters. The phosphorus speciation cycle of ruminant feed, feed ingredients, rumen digesta and manure will be investigated. It is anticipated that the issues surrounding obtaining data from biological

samples or the processing of data will be overcome, based on the experience with manure samples and phosphorus investigation which Dr. Peak has obtained.

### **Conclusions/Implications**

Phosphorus speciation in samples of grains, distillers' grains and manure from animals fed distillers' grains is clearly possible utilizing the PGM beamline at the Canadian Light Source. The phosphorus L-edge spectra indicates that there are changes in the form of phosphorus in each of these samples. However, additional data processing is necessary to understand if quantitative differences can be determined, in addition to the noted qualitative differences. This work will be published and will increase efforts to understand the behaviour of biological samples under analysis in a synchrotron soft x-ray beam line.

#### **Project 4. Use of synchrotron radiation FTIR Microspectroscopy to determine the distribution of cellulose, hemicellulose, and lignin in plant biomass.**

##### **Introduction**

Plant biomass is receiving increasing attention because it is annually renewable and herbaceous plants constitute the largest annual biomass stock (1549 million tons/year worldwide) (Kim et al., 2004; Allison et al. 2009). However, it is difficult to completely separate lignin from other cell wall polysaccharides. The lignin component is resistant to microbial degradation and constitutes a physical barrier to the extraction and utilization of the other components. Lignin is a complex aromatic heteropolymer formed from hydroxycinnamyl alcohol monomers (coumaryl, sinapyl, and coniferyl alcohols) differing in their degree of methoxylation (Boerjan et al. 2003). Wang (2002) noted that the chemical properties of plant cell wall compounds and the three dimensional cross-linked matrix of polysaccharides with lignin and other phenolic compounds limit digestion of cell wall polysaccharides by bacteria. Lignin deposition and composition are known to vary as a consequence of tissue-type, plant species, developmental stage and environmental stress (Campbell and Sederoff, 1996) and both parameters are known to affect thermal conversion efficiency (Demirbas, 2001).

A traditional method for analysis of lignin and cellulosic compounds is to check their total contents in a plant material, but this method is unable to check distribution and intensity of these compounds across plant tissue (Yu, 2007). Little research has been done to investigate the distribution of lignin and cellulosic compounds in intact plant stem material at a cellular level. Discovering the relationship between the chemical makeup of the cell wall and its digestion will help to understand the limitations they impose on the diets of animals and permit forages to be used more efficiently. We hypothesized that the synchrotron radiation-based FTIR

microspectroscopy (SFTIRM) can be used to examine the relative quantity of lignin and cellulosic compounds in the cell wall of the plant tissue. This technique takes advantage of synchrotron light brightness and is capable of exploring the molecular chemistry within microstructures of biological samples with high signal to noise ratio at ultraspatial resolutions as fine as 3-10  $\mu\text{m}$ . SFTIRM is able to identify molecular constituents in biological samples from their vibration spectra in mid-IR region. Simply put, when IR light passes through a sample, different functional groups and bonds of the biological components absorb (vibrate spectra) IR light independent of each other while some IR light pass through (Damiran and Yu, 2010). By measuring these different IR absorbed characteristics (molecular fingerprint: i.e., absorbed intensity, IR Unit; intensity ratios) it is possible to estimate the amount of each functional groups or the relative quantity of components (Yu et al., 2008) that exist in particular biological tissue. This information will help reveal the nature of cross-linkages within the cell wall and ultimately mechanisms for the release of glucose from lignocellulosic material. A recent work has shown that synchrotron radiation-based FTIR microspectroscopy can be used as a nondestructive tool to investigate the molecular structure of plant tissue at high-spatial resolution (Yu et al., 2008).

The hypothesis of this project is that the synchrotron radiation Fourier Transform Infrared (SR-FTIR) microspectroscopy can be used to examine the distribution of cellulose and lignin in the cell wall of plant material.

The objectives were to use SR-FTIR microspectroscopy to identify chemical differences in plant cell wall structure as wheat matures from the early milk stage to maturity:

- a. within a plant (i.e. intermodal differences along the plant stem) harvested at 3 stages of growth;
- b. between plants of different varieties harvested at 3 stages of growth;

## **Materials and Methods**

## **Plant Tissue**

Wheat seeds were obtained from the Department of Plant Sciences, University of Saskatchewan. Wheat plants (Canadian Western Red Spring and Canadian Western Durum Wheat) were grown in the Agriculture Green house (University of Saskatchewan, Canada). The plants consisted of three varieties of the red spring wheat under the class *Triticum aestivum* (CDC GO, hollow stem; McKenzie, intermediate stem and AC Eatonia, solid stem) and two varieties of the class *Durum turdigum* (CDC Verona, hollow stem and Golden ball, solid stem). The seeding rate was 1 plant per pot. Potting medium is Sunshine Professional Mix number 3 (SunGro Horticulture, British Columbia, Canada) and pot size was 15 cm in diameter x 18 cm in depth. The plants were grown in a controlled environment of 18 hours daylight and 6 hours darkness, at a temperature of 25 °C. Plants were watered as needed (approx. 500-750 ml every two days).

## **Proposed Method**

### ***Stem harvesting and sectioning***

The plant stems were harvested at three different growth stages, according to the Zadok two-digit code system for growth staging in wheat (Chang et al, 1974): Early milk (Zadok code 73), early dough (Zadok code 83) and mature stage Zadok code 92). Stems were uprooted from the soil and the stem length measured and noted. The variety, harvesting date, growth stage, number of tillers were also be noted. The number of nodes and internodes were determined according to Tottman, 1987 and noted. The nodes and internodes were numbered, starting from those closest to the roots. The stems were stored at 4oC prior to sectioning.

For the reported work, two varieties (a hollow stem, CDC GO and solid stem, AC Eatonia) from the *Triticum aestivum* class were chosen for cross-sectioning. This class was chosen because 90 % of the wheat grown in Canada falls under this class. The rest of the stems were stored at -20 °C for further analyses. Stems were being cross-sectioned along each internode for each of the growth stages (Early milk, early dough and maturity) (Fig. 1). Each sectioning was done midway through the internode. Two sections prepared for each part of the stem.

The stem sections were embedded in paraffin wax, and sectioned to a thickness of 6 microns, using a microtome. The sectioned samples were collected on BaF2 discs for transmission mode

analysis in Synchrotron radiation infrared micro-spectroscopy analyses at the Canadian Light Source. The sections were de-paraffinised through a series of washings with xylene/ethanol/water.

### **Synchrotron Light Source and SR-FTIR**

Experiments using the cross-sectioned stem samples were carried out at the Canadian Lightsource Incorporated (CLSI, Saskatoon, Canada) on the 01B1-1 mid infrared beam line, using a Hyperion microscope with programmable mapping stage, a Bruker IFS 66v/S Series IR spectrophotometer (Bruker Optics Inc., Billerica, MA, USA). Spectral data were collected in the mid-infrared range of 4000-800  $\text{cm}^{-1}$  at a resolution of 4  $\text{cm}^{-1}$  with 256 scans co-added, an aperture size of about 10  $\mu\text{m}$ . The background spectra were collected from a sample free area on the disc. Data was collected in transmission mode using a 36x IR objective. The microscope stage was continually being purged with dry  $\text{N}_2$  to minimize IR absorption from water vapor and  $\text{CO}_2$  in ambient air.

### **Data acquisition**

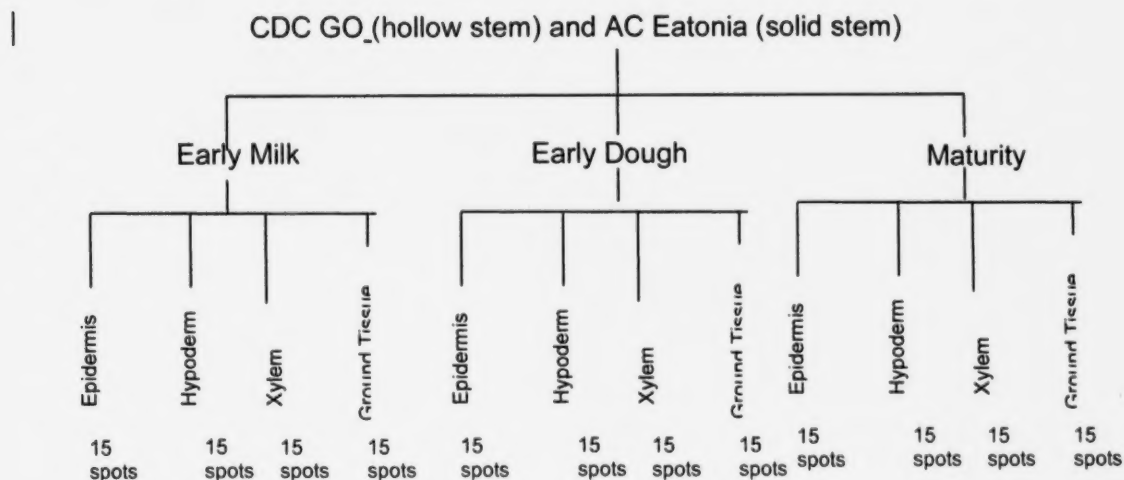
15 discrete spots were randomly selected from pure tissue components of the stem (the dermis, the hypodermis, the vascular bundles closest and furthest away from the epidermis and the ground tissue) for each section (Figure 4.1).

### **Data Analysis**

Each sample spectra was corrected by subtracting the background spectrum and displayed in absorbance units. Automatic baseline correction and normalization will be done using OPUS software (OPUS 6.5) to generate the final spectra. Spectra will be examined for quality in terms of the absorption range. Absorbance intensities of the functional groups - 1250  $\text{cm}^{-1}$  for cellulosic material for example will be analysed by spectral peak heights. The ratio of cellulose to lignin SR-FTIR absorbance intensity was calculated as:

Ratio = SR-FTIR absorbance intensity of lignin/SR-FTIR absorbance intensity of cellulosic compounds. Statistical analyses was performed using the t-test at 95 % confidence level using the SAS Institute , Inc.

**Figure 4.1: Flow chart of work plan at the synchrotron**



## Results and Discussion

This is the first study to our knowledge that has attempted to determine the relative concentration of lignin and cellulosic compounds in the plant cell wall using SFTIR microspectroscopy. A typical absorption spectrum from a section of wheat stem tissue is shown in Figures 4.2 and 4.3. The absorption (IR absorbed intensity; IR Unit) band peaks at ca. 1510  $\text{cm}^{-1}$  and ca. 1240  $\text{cm}^{-1}$  correspond to lignin and cellulosic compounds, respectively (Wetzel et al, 1998; Wetzel and LeVine, 2000; Yu, 2007). Infrared absorption (peak area or peak height) size is correlated with concentration, therefore, in this report; we are reporting IR absorbance intensity as relative concentration.

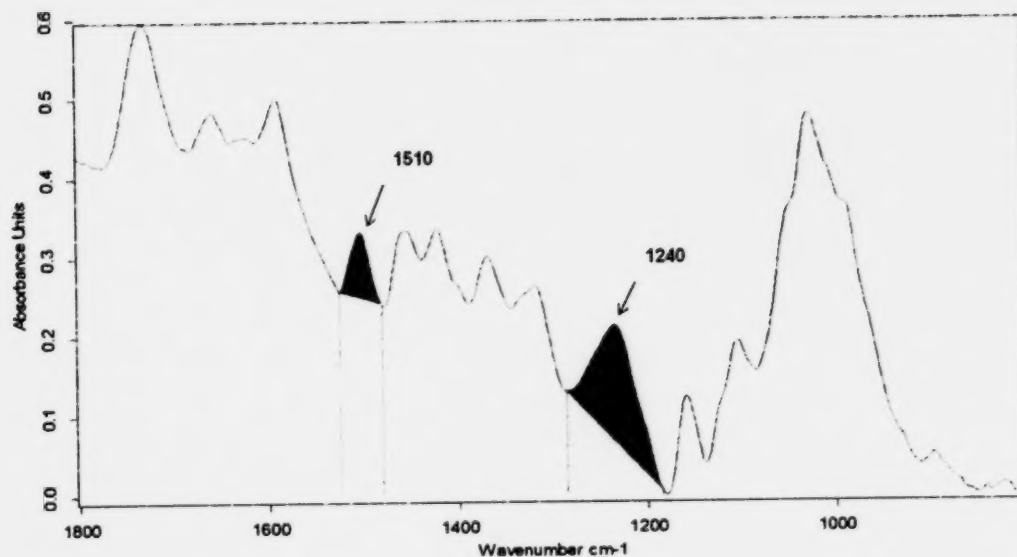


**Figure 4.2:** Typical spectrum of wheat stem in fingerprint region, ca. 1800-800  $\text{cm}^{-1}$  in the mid-IR region, illustrating the calculation of peak area attributed to the absorption peaks of aromatic lignin (ca. 1530-1480  $\text{cm}^{-1}$ ) and cellulosic compounds (ca. 1285-1185  $\text{cm}^{-1}$ ).

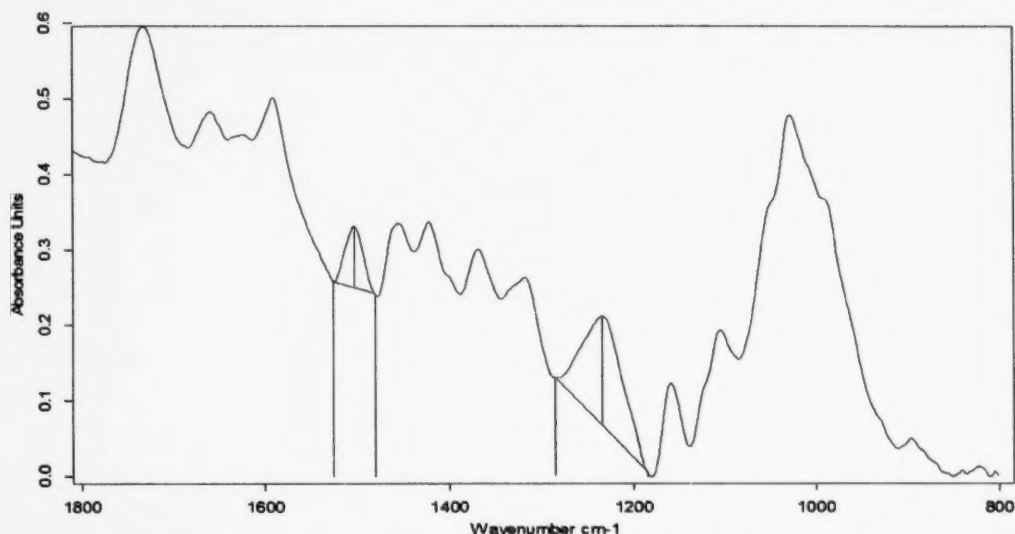


**Figure 4.3:** Typical spectrum of wheat stem in fingerprint region, ca. 1800-800  $\text{cm}^{-1}$  in the mid-IR region, illustrating the calculation of peak height for the absorption peaks of lignin (ca.  $\sim 1510 \text{ cm}^{-1}$ ) and cellulosic compounds (ca.  $\sim 1240 \text{ cm}^{-1}$ ).

Wheat stems are composed of four main tissue regions. These include the epidermis, hypoderm, xylem, and ground tissue. These different types have evolved as adaptations to the environment in which vascular plants survive and are specialized for one or more functions (Beck, 2005). Therefore we have analyzed and reported each tissue separately. For all tissues there were significant interactions ( $P < 0.001$ ) between the variety, harvesting stage, and sampling location for both lignin and cellulosic compounds and their ratio. These interactions are presented in Tables 1.1 to 1.3 in Appendix A). In order to explain these interactions we will discuss each tissue separately and focus on the ratio of lignin to cellulosic compounds as this measurement is not influenced by sample thickness. Note in this report we refer to cellulosic compounds which refers to both cellulose and hemicellulose as it is not possible to distinguish the two in terms of IR absorbance.

**Epidermis Tissue:** The epidermis is the outermost covering of the stem represented by a single layer of compactly arranged, barrel-shaped parenchyma cells. Intercellular spaces are absent while a cuticle may be present (Beck, 2005). Figure 4.4 shows the interaction between stage of cutting, sampling location and variety on the ratio of lignin to cellulosic compounds (L:C) absorbance IR units in the epidermis for both peak area and height.

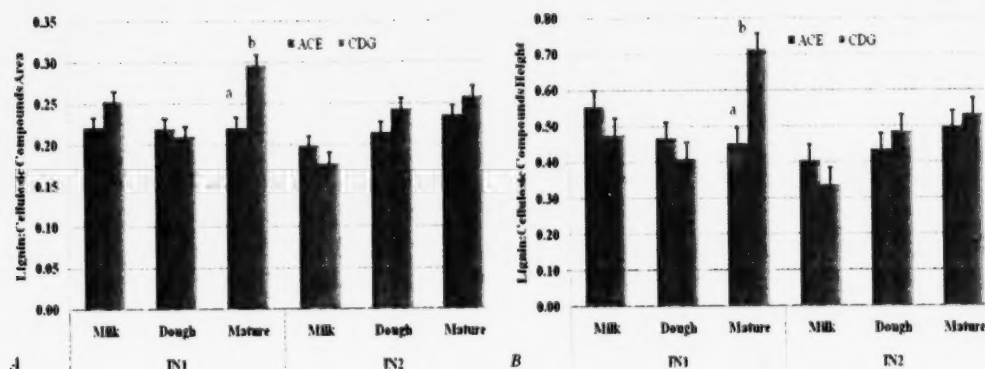


**Figure 4.4.** The effects of wheat variety on lignin to cellulosic compounds relative concentration (IR Unit) peak area ratio (A) and peak height ratio (B) in wheat stem epidermis tissue wall detected by SFTIRM: Wheat variety: ACE, AC Eatonia, CDG, CDC GO. Column with different letters within location (IN1, Internode 1; IN2, Internode 2) and within growth stage are significantly different at  $P < 0.05$ .

Note that for the solid stem variety AC Eatonia, the peak area ratio is relatively constant across stage of maturity for the more mature part of the stem (internode one). However, the hollow stem variety tends to have a greater ratio at the milk and maturity stages than AC Eatonia in this section of the stem; this is particularly evident in internode 1 of the mature plant. In the less mature part of the stem (i.e. internode 2), the L:C ratio increases in both varieties with stage of maturity and tends to be greater for the hollow stem CDC GO variety. This increasing concentration of lignin as the plant matures and as we move up the stem likely contributes biologically to plant stability and aids in allowing the plant to stand upright and thus facilitate

growth. Similarly, biologically it stands to reason that a hollow stemmed variety would require a greater concentration of lignin in the cell wall of the stem relative to a solid stemmed variety in order to facilitate plant stability. Similar results were seen when L:C peak heights were considered (Figure 4.4b).

**Hypoderm Tissue:** The hypoderm is a region that lies immediately below the epidermis. It is represented by a few layers of compactly arranged lignified cellulose-rich sclerenchyma cells (Hamman et al, 2005). Sclerenchyma cells are the main supporting cells of a plant (Beck, 2005). With regard to the L:C pear area ratio, we see very similar results to the epidermis. In the more mature internode 1, the ratio is similar across stage of maturity for the solid stem AC Eatonia but increases linearly for the hollow stem variety CDC GO as stage of maturity advances (Figure 4.5). When we move to the less mature, more actively growing tissue, the L:C ratio for both plants increases linearly with stage of maturity. Thus the more mature the plant at harvest the greater the ratio of lignin to cellulosic compounds in the 2<sup>nd</sup> internode, which again from a biological perspective likely provides the mature plant with greater stability and structural integrity (Figure 4.5a). Again similar results were noted when peak height was used to determine the L:C ratio (Figure 4.5b).

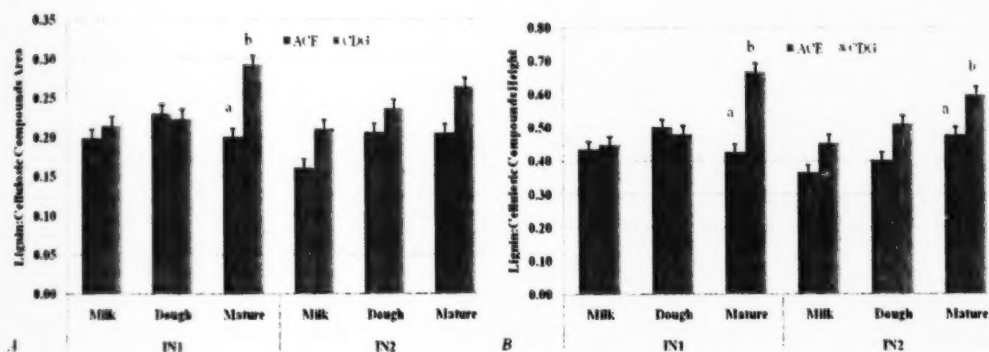


**Figure 4.5.** The effects of wheat variety on lignin to cellulosic compounds relative concentration (IR Unit) peak area ratio (A) and peak height ratio (B) in wheat stem hypoderm tissue wall detected by SFTIRM: Wheat variety: ACE, AC Eatonia, CDG, CDC GO. Column with different letters within location (IN1, Internode 1; IN2, Internode 2) and within growth stage are significantly different at  $P < 0.05$ .

**Xylem:** Each vascular bundle has a covering called the bundle sheath which is formed by a single layer of sclerenchyma cells (Hamman et al., 2005). The vascular bundle encloses both xylem and phloem. Xylem forms on the inside of the bundle. The vascular bundles are described as conjoint, collateral, and closed (Beck, 2005). In the xylem, there are two lignified, cellulose rich metaxylem (so called monkey eyes) and two protoxylem vessels arranged in 'the shape of 'Y' (Hamman et al., 2005). We selected the metaxylem tissue for investigation. Figure 4.6 shows the interaction between stage of cutting, sampling location and variety on the ratio of lignin to cellulosic compounds (L:C) in the xylem for both peak area and height. As opposed to the outer 2 tissue layers, the L:C ratio was fairly consistent across the milk and dough stages of maturity for varieties, however the L:C ratio was greater ( $P < 0.05$ ) for CDC GO relative to AC Eatonia in the mature plants, a response that was common across both internodes, particularly when peak height was measured (Figure 4.6b). Again greater lignification of the cell wall of a hollow

stemmed variety (CDC GO) relative to a solid stem variety (AC Eatonia) would provide greater support and stability to the hollow stemmed plant.

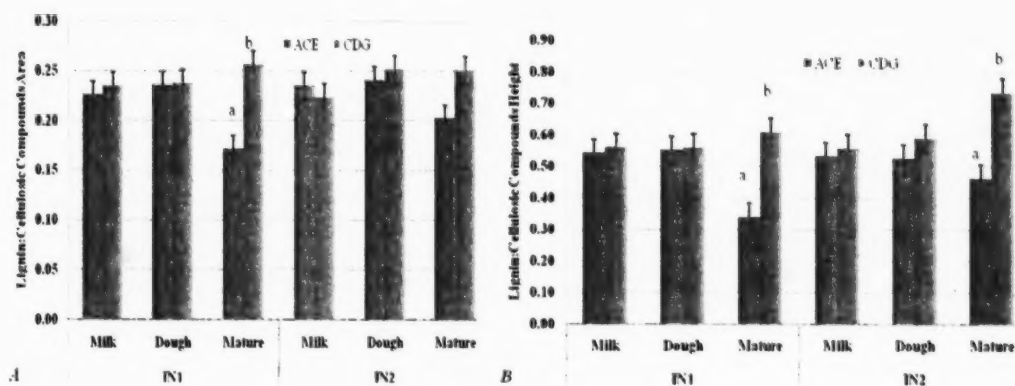


**Figure 4.6.** The effects of wheat variety on lignin to cellulosic compounds relative concentration (IR Unit) peak area ratio (A) and peak height ratio (B) in wheat stem xylem tissue wall detected by SFTIRM: Wheat variety: ACE, AC Eatonia, CDG, CDC GO. Column with different letters within location (IN1, Internode 1; IN2, Internode 2) and within growth stage are significantly different at  $P < 0.05$ .

**Ground Tissue:** Ground tissue is a major component of the stem. It is undifferentiated. The ground tissue is represented by several layers of loosely arranged lignified parenchyma cells enclosing prominent intercellular spaces (Hamman et al., 2005). Spectral data collected from the ground tissues was of poor quality due to the minimal thickness of the cell walls (< 2 microns) and minimal composition of lignin and cellulosic compounds and resulted in a great deal of noise and were not suitable for analysis.

The interaction showing differences between the hollow stemmed variety (CDC GO) and the solid stemmed variety (AC Eatonia) in terms of L:C ratio as the plants mature is shown in

Figures 4.7 to 4.10. For the hollow stemmed variety, CDC GO the L:C ratio generally increase with maturity irregardless of tissue studied (Figures 4.7 & 4.8). in contract the solid stem variety tends to maintain a constant ratio as L:C ratio increases (Figures 4.9 & 4.10).

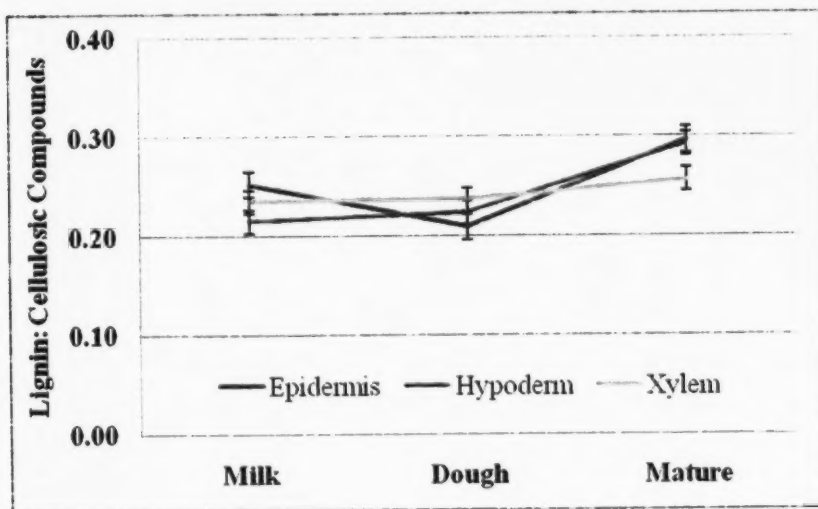


**Figure 4.7.** The effects of growth stage on lignin:cellulosic compounds peak area ratio in internode 1 of CDC GO wheat stem.

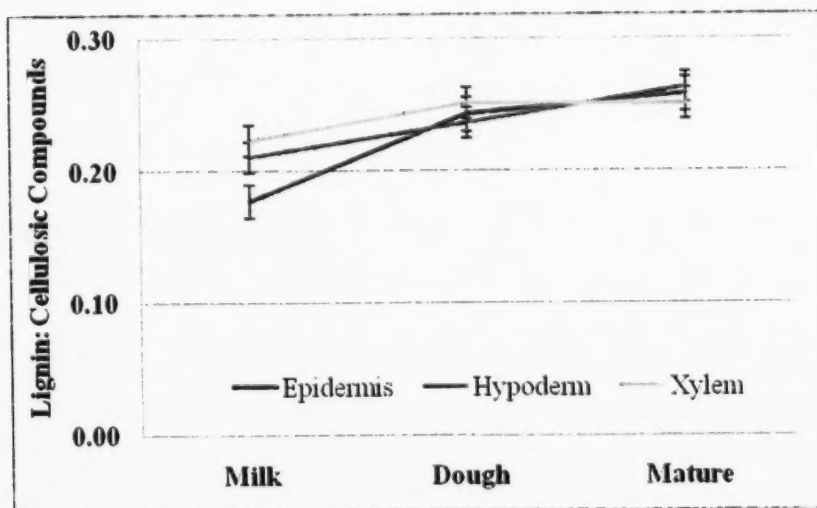


**Figure 4.8.** The effects of growth stage on lignin:cellulosic compounds peak area ratio in internode 2 of CDC GO wheat stem

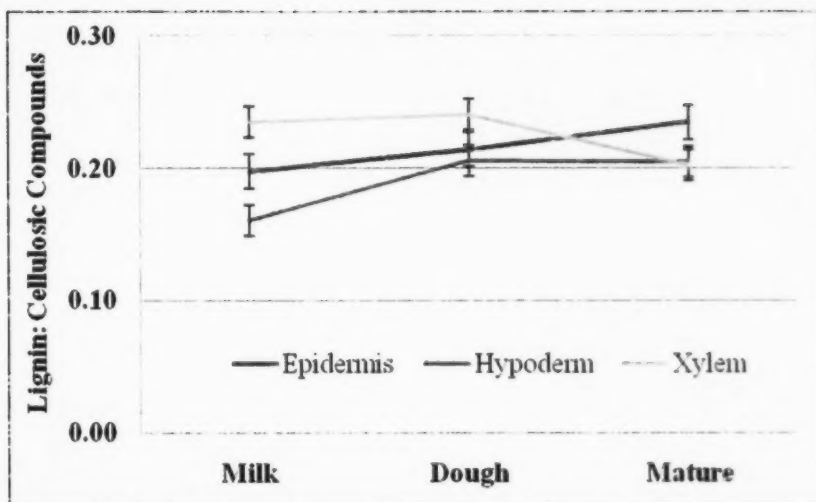


**Figure 4.9.** The effects of growth stage on lignin:cellulosic compounds peak area ratio in internode 2 of AC Eatonia wheat stem

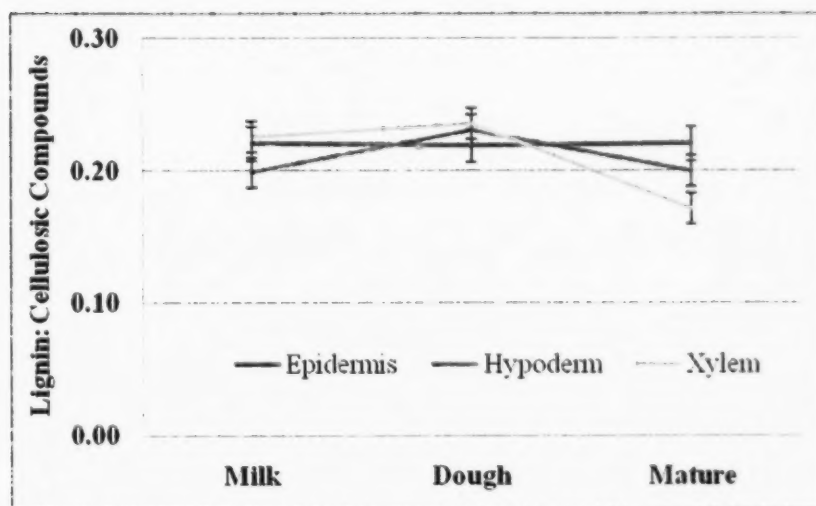


**Figure 4.10.** The effects of growth stage on lignin:cellulosic compounds peak area ratio in internode 1 of AC Eatonia wheat stem

**Conclusions:**

Synchrotron FTIRM can be used to reveal spectral characteristics of aromatic lignin and cellulosic compounds and their spectral intensity ratio in the tissue cell wall of the wheat stem. The studies suggested that the lignin to cellulosic compounds ratio may be the best parameter to characterize the degree of lignifications of plant stems of different varieties at different growth stages and that this technique can be used to identify components of the plant associated with nutrient utilization by ruminants. Future research in this should focus on development of appropriate model for assessing nutrient utilization in the rumen that can be correlated with detailed analysis on chemical composition that is now available through appropriate beam lines of the Canadian Light Source.

**References:** Available upon request.

## Appendix A

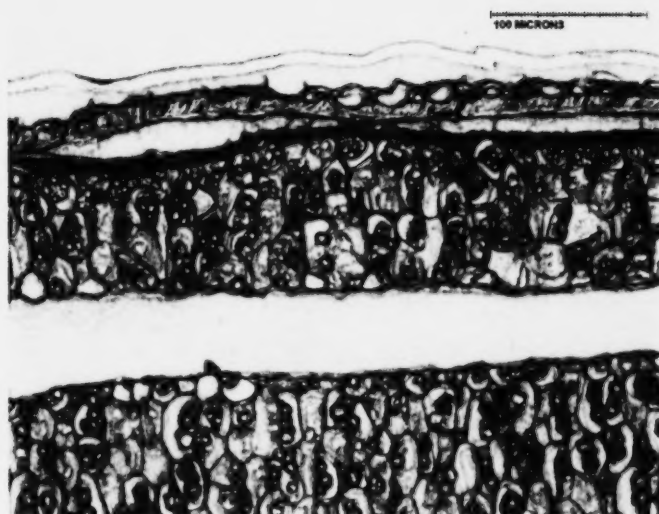


**Figure 1.** Photomicrograph of cross-section of flaxseed (cv. vimy) seed showing the intrinsic structure – the pericarp (outside of seed coat), seed coat, aleurone and endosperm (magnification: 10×20) [Picture was taken using Olympus Compound Microscopy with digital camera Model BX41TF, Olympus Optical Co. Ltd]

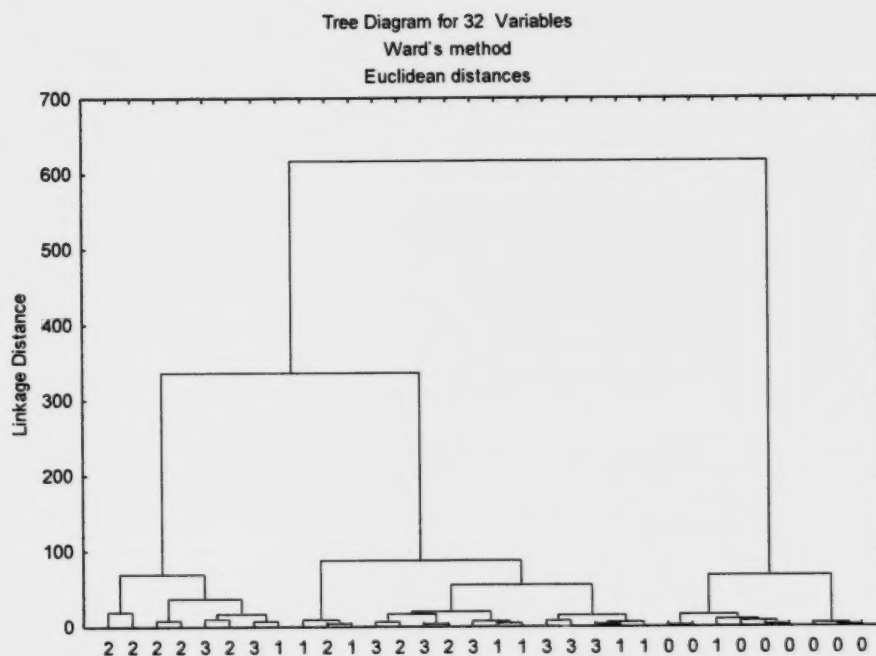
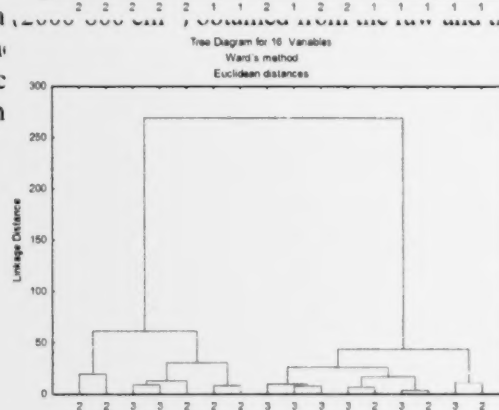
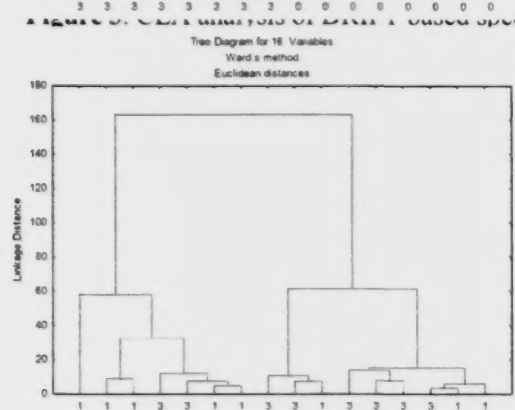
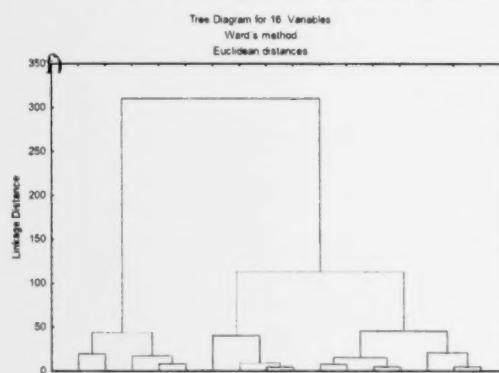
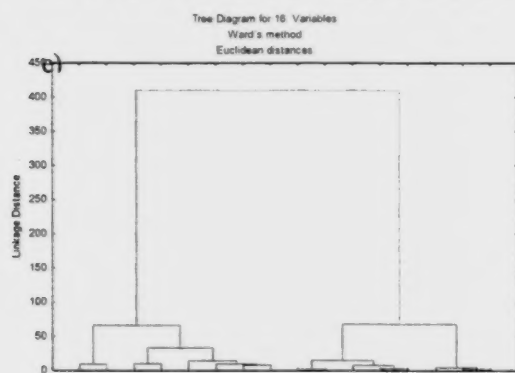
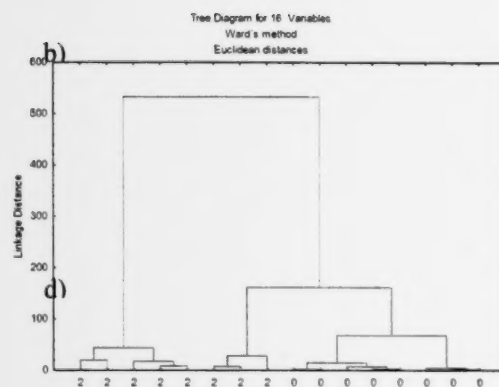
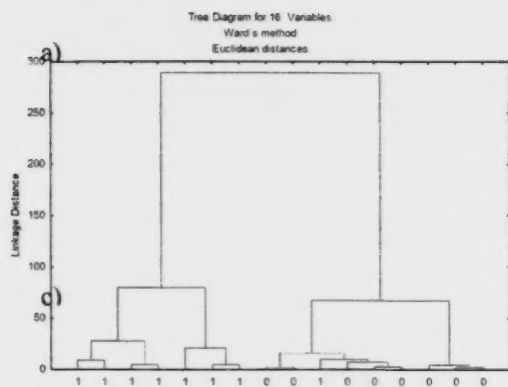


**Figure 2.** CLA analysis of DRIFT based spectra ( $2000\text{-}800\text{ cm}^{-1}$ ) obtained from the raw and the autoclaved flaxseed samples (0 = control/raw, 1 =  $120^{\circ}\text{C}$  for 20 min, 2 =  $120^{\circ}\text{C}$  for 40 min, 3 =  $120^{\circ}\text{C}$  for 60 min) [CLA = Hierarchical cluster analysis, DRIFT = Diffuse Reflectance Infrared Fourier Transform]



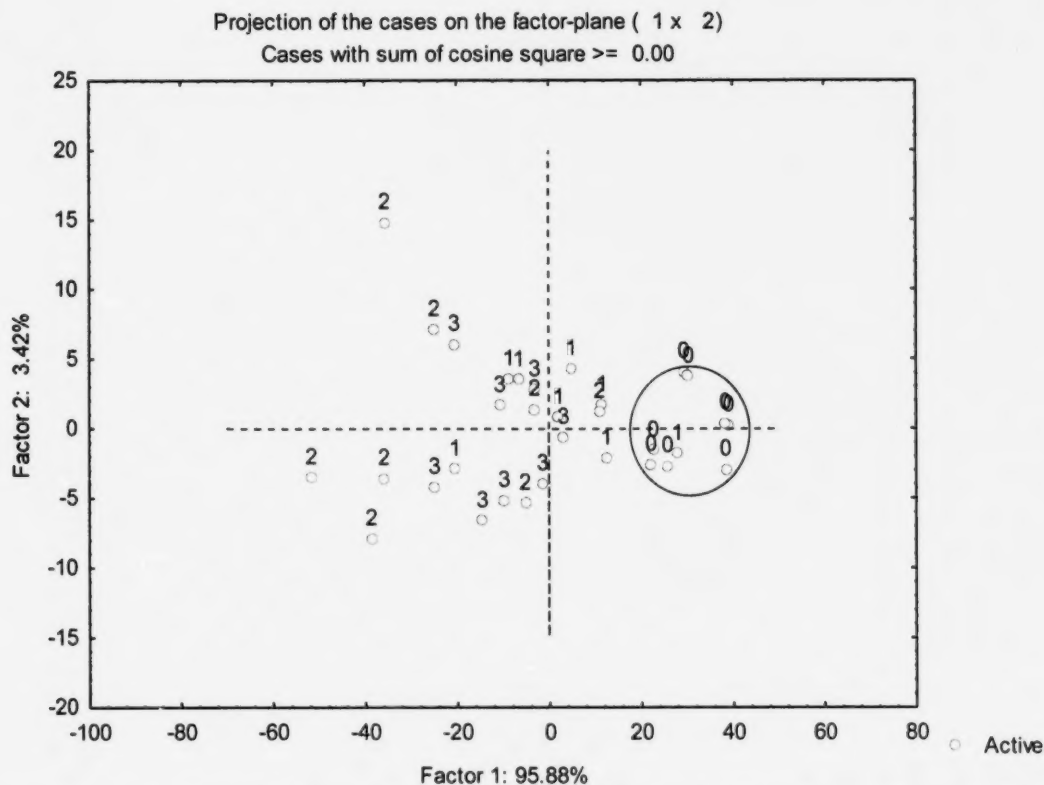


**Figure 4.** PCA analysis of DRIFT based spectra ( $2000\text{-}800\text{ cm}^{-1}$ ) obtained from the raw and the autoclaved flaxseed samples (0 = control/raw, 1 =  $120^{\circ}\text{C}$  for 20 min, 2 =  $120^{\circ}\text{C}$  for 40 min, 3 =  $120^{\circ}\text{C}$  for 60 min) [PCA = Principal components analysis, DRIFT = Diffuse Reflectance Infrared Fourier Transform]

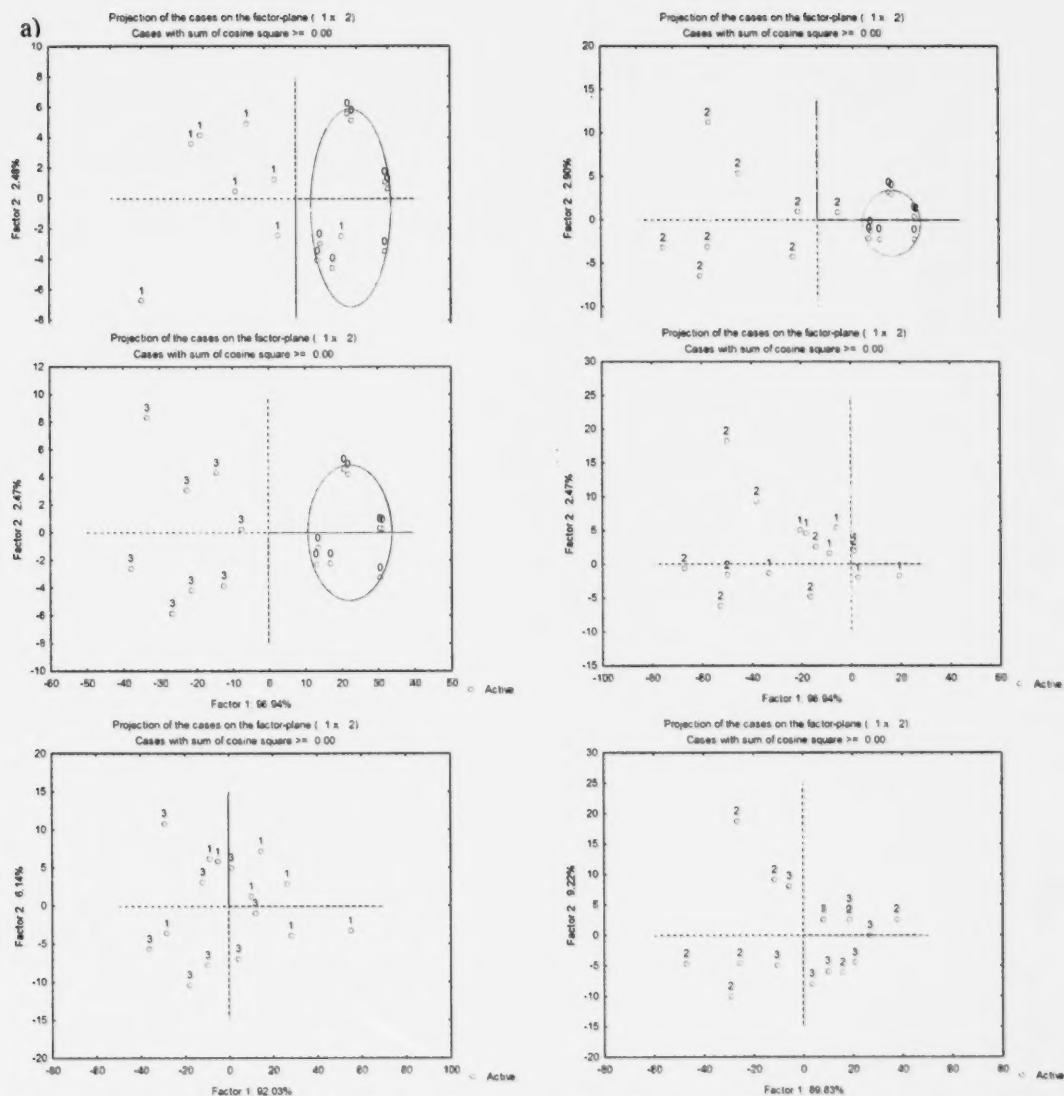


**Figure 5.** PCA analysis of DRIFT based spectra ( $2000\text{--}800\text{ cm}^{-1}$ ) obtained from the raw and the autoclaved flaxseed samples. Comparisons of the separate treatments. (a = 0 vs. 1, b = 0 vs. 2, c = 0 vs. 3, d = 1 vs. 2, e = 1 vs. 3, f = 2 vs. 3) (0 = control/raw, 1 =  $120^{\circ}\text{C}$  for 20 min, 2 =  $120^{\circ}\text{C}$  for 40 min, 3 =  $120^{\circ}\text{C}$  for 60 min) [PCA = Principal components analysis, DRIFT = Diffuse Reflectance Infrared Fourier Transform]

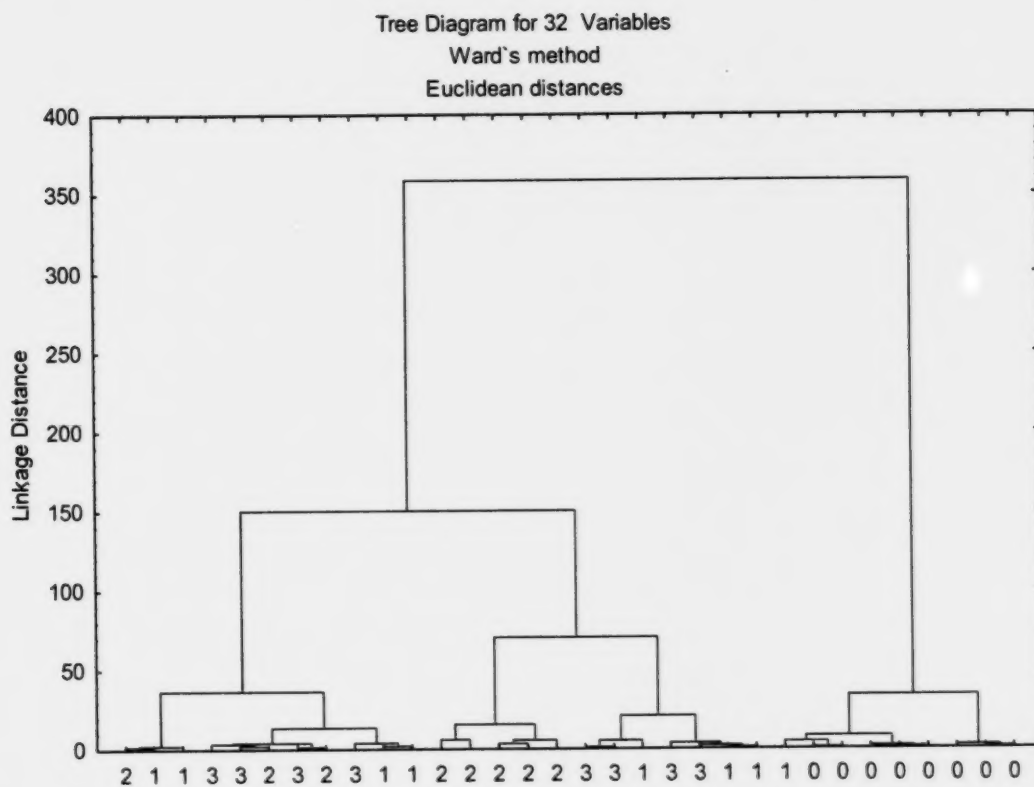


**Figure 6.** CLA analysis of DRIFT based spectra of the amide I and II region ( $1715\text{-}1485\text{ cm}^{-1}$ ) obtained from the raw and the autoclaved flaxseed samples (0 = control/raw, 1 =  $120^{\circ}\text{C}$  for 20 min, 2 =  $120^{\circ}\text{C}$  for 40 min, 3 =  $120^{\circ}\text{C}$  for 60 min) [CLA = Hierarchical cluster analysis, DRIFT = Diffuse Reflectance Infrared Fourier Transform]

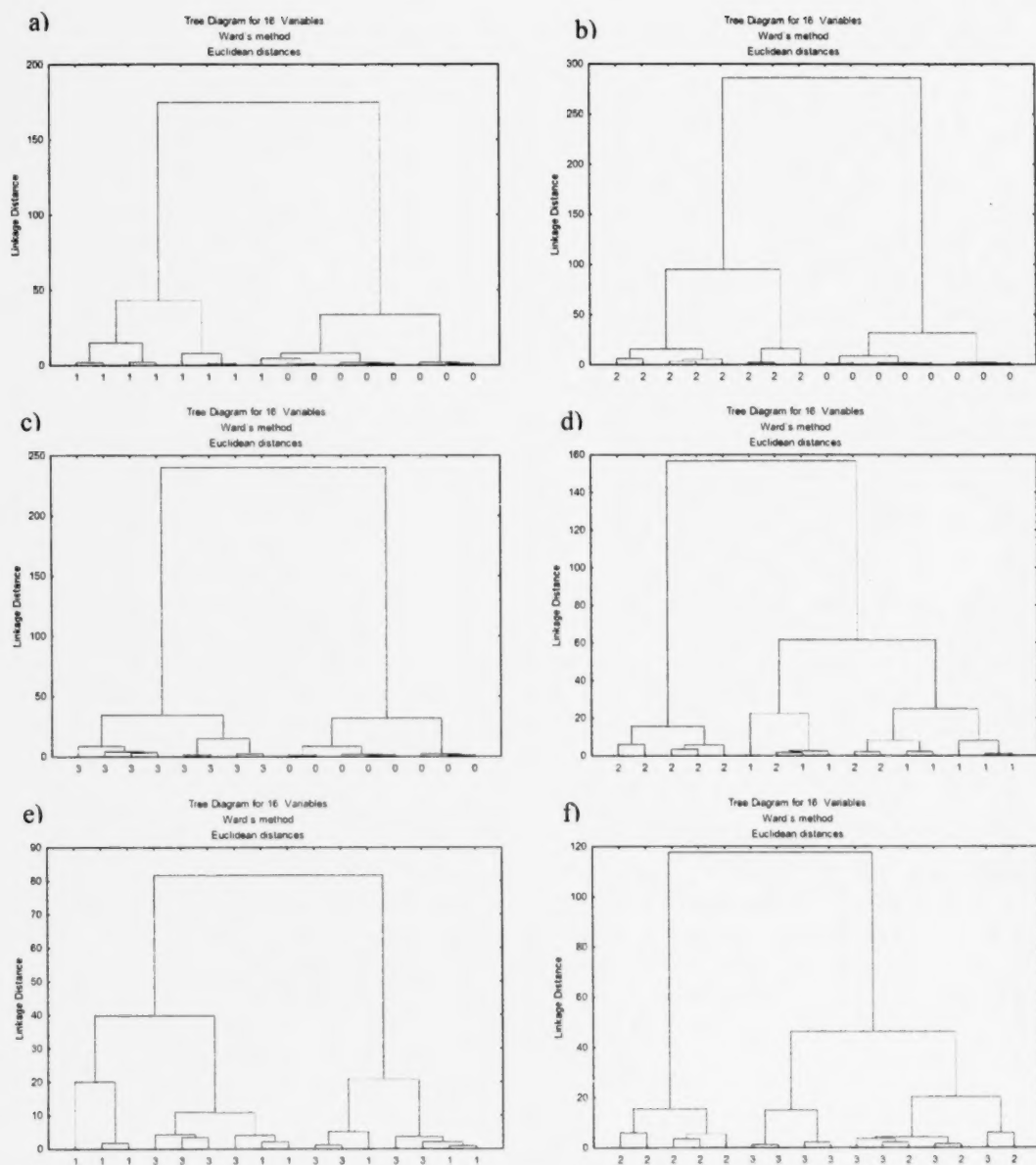


**Figure 7.** CLA analysis of DRIFT based spectra of the amide I and II region ( $1715\text{--}1485\text{ cm}^{-1}$ ) obtained from the raw and the autoclaved flaxseed samples. Comparisons of the separate treatments. (a = 0 vs. 1, b = 0 vs. 2, c = 0 vs. 3, d = 1 vs. 2, e = 1 vs. 3, f = 2 vs. 3) (0 = control/raw, 1 =  $120^{\circ}\text{C}$  for 20 min, 2 =  $120^{\circ}\text{C}$  for 40 min, 3 =  $120^{\circ}\text{C}$  for 60 min) [CLA = Hierarchical cluster analysis, DRIFT = Diffuse Reflectance Infrared Fourier Transform].

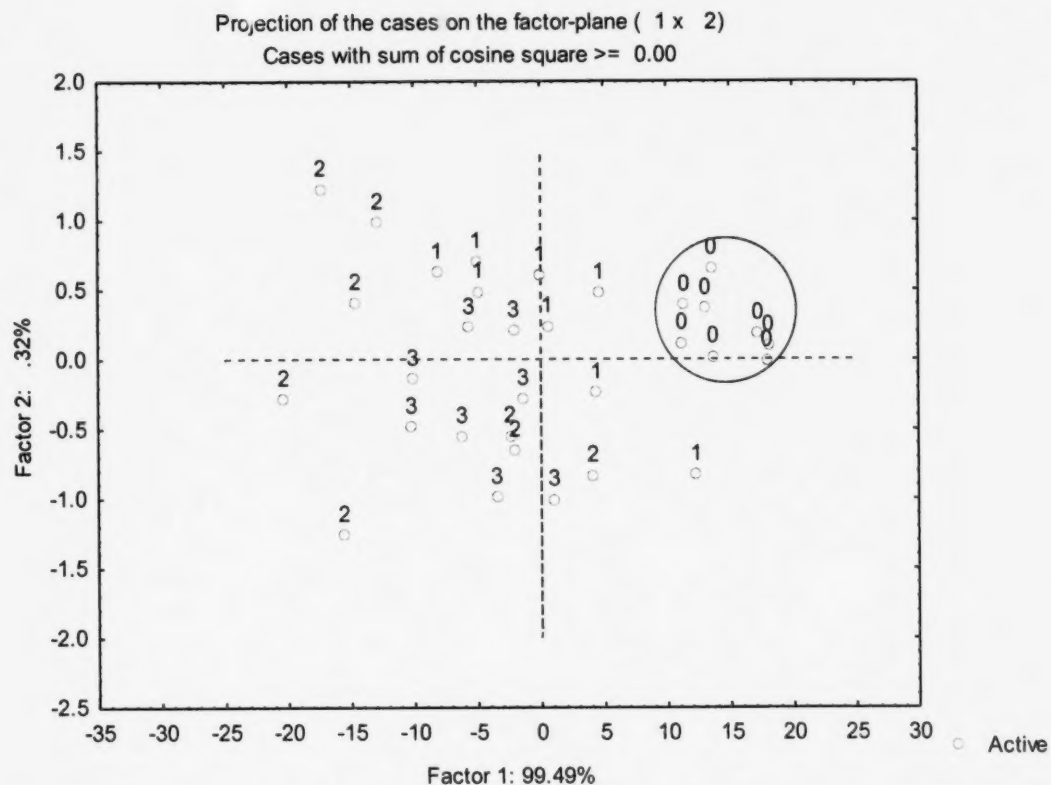


**Figure 8** PCA analysis of DRIFT based spectra for the amide I and II region ( $1715\text{--}1485\text{ cm}^{-1}$ ) obtained from the raw and the autoclaved flaxseed samples (0 = control/raw, 1 =  $120^{\circ}\text{C}$  for 20 min, 2 =  $120^{\circ}\text{C}$  for 40 min, 3 =  $120^{\circ}\text{C}$  for 60 min) [PCA = Principal components analysis, DRIFT = Diffuse Reflectance Infrared Fourier Transform]

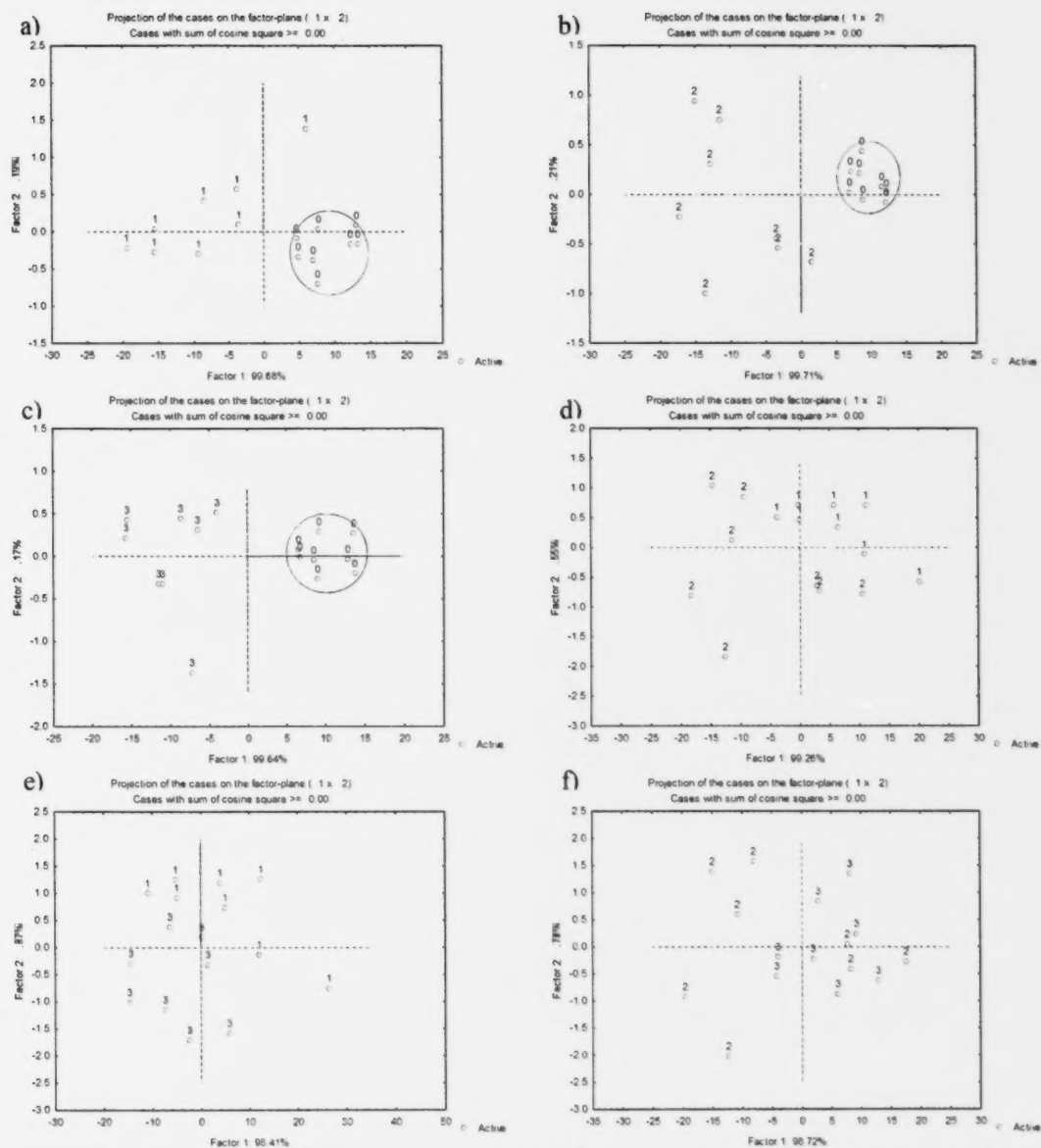


**Figure 9.** PCA analysis of DRIFT based spectra for the amide I and II region ( $1715\text{--}1485\text{ cm}^{-1}$ ) obtained from the raw and the autoclaved flaxseed samples. Comparisons of the separate treatments. (a = 0 vs. 1, b = 0 vs. 2, c = 0 vs. 3, d = 1 vs. 2, e = 1 vs. 3, f = 2 vs. 3) (0 = control/raw, 1 =  $120^{\circ}\text{C}$  for 20 min, 2 =  $120^{\circ}\text{C}$  for 40 min, 3 =  $120^{\circ}\text{C}$  for 60 min) [PCA = Principal components analysis, DRIFT = Diffuse Reflectance Infrared Fourier Transform].

**Appendix B: Table 1.1.** The effects of wheat variety, growth stage, and sampled location on relative concentration of lignin and cellulosic compounds in internode epidermis tissue wall detected by synchrotron radiation-based fourier transform infrared (IR) microspectroscopy (SFTIRM)

| Variety              | Stage  | Location    | SFTIRM IR Absorption intensity (IR Unit) |                        |                            |                        |                 |             |
|----------------------|--------|-------------|------------------------------------------|------------------------|----------------------------|------------------------|-----------------|-------------|
|                      |        |             | Lignin (L)                               |                        | Cellulosic compounds (C)   |                        | Ratio of L to C |             |
|                      |        |             | Peak area                                | Peak height            | Peak area                  | Peak height            | Peak area       | Peak height |
|                      |        |             | 1530-1480 cm <sup>-1</sup>               | ~1510 cm <sup>-1</sup> | 1285-1185 cm <sup>-1</sup> | ~1240 cm <sup>-1</sup> |                 |             |
| AC Eatonia           | Milk   | Internode 1 | 2.50 a                                   | 0.12 a                 | 11.05 a                    | 0.22 a                 | 0.22 bcde       | 0.55 ab     |
|                      |        | Internode 2 | 1.49 de                                  | 0.06 bc                | 6.73 c                     | 0.14 d                 | 0.20 cfg        | 0.40 b      |
|                      | Dough  | Internode 1 | 1.65 cde                                 | 0.07 bc                | 7.26 c                     | 0.15 d                 | 0.22 bcde       | 0.47 b      |
|                      |        | Internode 2 | 1.56 de                                  | 0.06 bc                | 7.30 c                     | 0.15 d                 | 0.21 bcde       | 0.43 b      |
|                      | Mature | Internode 1 | 2.11 abc                                 | 0.08 abc               | 9.91 ab                    | 0.20 abc               | 0.22 bcde       | 0.45 b      |
|                      |        | Internode 2 | 2.35 abc                                 | 0.10 ab                | 10.10 ab                   | 0.20 ab                | 0.23 bde        | 0.50 ab     |
| CDC GO               | Milk   | Internode 1 | 1.42 cde                                 | 0.05 bc                | 5.71 c                     | 0.12 d                 | 0.25 bc         | 0.48 b      |
|                      |        | Internode 2 | 1.10 e                                   | 0.04 c                 | 6.14 c                     | 0.13 cd                | 0.18 eg         | 0.34 b      |
|                      | Dough  | Internode 1 | 1.22 de                                  | 0.05 c                 | 5.89 c                     | 0.12 d                 | 0.21 df         | 0.41 b      |
|                      |        | Internode 2 | 1.62 bcd                                 | 0.07 bc                | 6.55 bc                    | 0.13 bcd               | 0.24 bc         | 0.48 b      |
|                      | Mature | Internode 1 | 1.62 abcd                                | 0.08 abc               | 5.68 c                     | 0.11 d                 | 0.30 a          | 0.71 a      |
|                      |        | Internode 2 | 1.30 cde                                 | 0.05 c                 | 4.91 c                     | 0.10 d                 | 0.26 b          | 0.53 ab     |
| SEM                  |        |             | 0.187                                    | 0.010                  | 0.791                      | 0.020                  | 0.010           | 0.046       |
| Statistical analysis |        |             | -----P value-----                        |                        |                            |                        |                 |             |
| Variety (V)          |        |             | 0.0662                                   | 0.0388                 | 0.0403                     | 0.0419                 | 0.2386          | 0.459       |
| Stage (S)            |        |             | <.0001                                   | 0.0171                 | 0.0095                     | 0.0277                 | <.0001          | 0.001       |
| Location (L)         |        |             | 0.0033                                   | 0.0067                 | 0.0113                     | 0.0088                 | 0.0002          | 0.014       |
| V*S                  |        |             | 0.0001                                   | 0.0266                 | <.0001                     | <.0001                 | <.0001          | 0.002       |
| V*L                  |        |             | 0.1095                                   | 0.3046                 | 0.0031                     | 0.0051                 | 0.0078          | 0.502       |
| S*L                  |        |             | <.0001                                   | 0.0030                 | 0.0004                     | 0.0030                 | <.0001          | 0.031       |
| V*S*L                |        |             | <.0001                                   | 0.0004                 | <.0001                     | <.0001                 | <.0001          | 0.028       |

SEM = Pooled standard error of means. Means in the same column with different letters are significant different at  $P < 0.05$  level using the Turkey-Kramer procedure

**Appendix B: Table 1.2.** The effects of wheat variety, growth stage, and sampled location on relative concentration of lignin and cellulosic compounds in internode hypoderm tissue wall detected by synchrotron radiation-based fourier transform infrared (IR) microspectroscopy (SFTIRM)

| Variety              | Stage  | Location    | SFTIRM IR Absorption intensity (IR Unit) |                        |                            |                        |                 |             |
|----------------------|--------|-------------|------------------------------------------|------------------------|----------------------------|------------------------|-----------------|-------------|
|                      |        |             | Lignin (L)                               |                        | Cellulosic compounds (C)   |                        | Ratio of L to C |             |
|                      |        |             | Peak area                                | Peak height            | Peak area                  | Peak height            | Peak area       | Peak height |
|                      |        |             | 1530-1480 cm <sup>-1</sup>               | ~1510 cm <sup>-1</sup> | 1285-1185 cm <sup>-1</sup> | ~1240 cm <sup>-1</sup> |                 |             |
| AC Eatonia           | Milk   | Internode 1 | 2.22 abc                                 | 0.11 ab                | 11.47 a                    | 0.25 a                 | 0.20 c          | 0.43 cde    |
|                      |        | Internode 2 | 1.75 def                                 | 0.08 cd                | 10.91 ab                   | 0.22 abc               | 0.16 d          | 0.36 f      |
|                      | Dough  | Internode 1 | 1.98 abcde                               | 0.09 cd                | 8.53 cd                    | 0.18 def               | 0.23 bc         | 0.50 bc     |
|                      |        | Internode 2 | 2.10 abcd                                | 0.09 cd                | 9.86 abcd                  | 0.21 bcd               | 0.21 c          | 0.40 ef     |
|                      | Mature | Internode 1 | 1.61 ef                                  | 0.07 cd                | 8.07 cd                    | 0.16 ef                | 0.20 c          | 0.42 def    |
|                      |        | Internode 2 | 1.85 abcde                               | 0.08 cd                | 9.34 bcd                   | 0.18 bcde              | 0.20 c          | 0.48 cd     |
| CDC GO               | Milk   | Internode 1 | 1.66 bcf                                 | 0.07 bd                | 7.72 abcd                  | 0.16 abcde             | 0.21 c          | 0.45 cdef   |
|                      |        | Internode 2 | 1.82 abcde                               | 0.08 abcd              | 8.68 abc                   | 0.18 abde              | 0.21 cd         | 0.45 cdef   |
|                      | Dough  | Internode 1 | 1.50 cf                                  | 0.06 d                 | 6.63 d                     | 0.13 cf                | 0.22 c          | 0.48 cde    |
|                      |        | Internode 2 | 1.96 abde                                | 0.08 abc               | 8.30 abcd                  | 0.17 abcde             | 0.24 bc         | 0.51 cde    |
|                      | Mature | Internode 1 | 2.07 ade                                 | 0.09 ac                | 7.27 bcd                   | 0.14 cf                | 0.29 a          | 0.67 a      |
|                      |        | Internode 2 | 1.96 abde                                | 0.09 abc               | 7.38 abcd                  | 0.14 bcde              | 0.26 bc         | 0.60 b      |
| SEM                  |        |             | 0.227                                    | 0.009                  | 0.891                      | 0.020                  | 0.012           | 0.025       |
| Statistical analysis |        |             | -----P value-----                        |                        |                            |                        |                 |             |
| Variety (V)          |        |             | 0.7740                                   | 0.5555                 | 0.1534                     | 0.1561                 | 0.0443          | 0.0359      |
| Stage (S)            |        |             | 0.9520                                   | 0.6117                 | <.0001                     | <.0001                 | <.0001          | <.0001      |
| Location (L)         |        |             | 0.1860                                   | 0.4298                 | 0.0005                     | 0.0073                 | 0.0008          | 0.0028      |
| V*S                  |        |             | <.0001                                   | <.0001                 | 0.0093                     | 0.0090                 | <.0001          | <.0001      |
| V*L                  |        |             | 0.0450                                   | 0.0028                 | 0.6021                     | 0.1812                 | 0.1085          | 0.0927      |
| S*L                  |        |             | 0.0020                                   | 0.0005                 | 0.0631                     | 0.0027                 | 0.2944          | 0.3504      |
| V*S*L                |        |             | 0.0003                                   | <.0001                 | 0.0504                     | 0.0120                 | 0.0001          | <.0001      |

SEM = Pooled standard error of means. Means in the same column with different letters are significant different at  $P < 0.05$  level using the Turkey-Kramer procedure

**Appendix B: Table 1.3.** The effects of wheat variety, growth stage, and sampled location on relative concentration of lignin and cellulosic compounds in internode xylem tissue wall detected by synchrotron radiation-based fourier transform infrared (IR) microspectroscopy (SFTIRM)

|                      |        |             | SFTIRM IR Absorption intensity (IR Unit) |                        |                            |                        |                 |             |
|----------------------|--------|-------------|------------------------------------------|------------------------|----------------------------|------------------------|-----------------|-------------|
| Variety              | Stage  | Location    | Lignin (L)                               |                        | Cellulosic compounds (C)   |                        | Ratio of L to C |             |
|                      |        |             | Peak area                                | Peak height            | Peak area                  | Peak height            | Peak area       | Peak height |
|                      |        |             | 1530-1480 cm <sup>-1</sup>               | ~1510 cm <sup>-1</sup> | 1285-1185 cm <sup>-1</sup> | ~1240 cm <sup>-1</sup> |                 |             |
| AC Eatonia           | Milk   | Internode 1 | 1.68 a                                   | 0.08                   | 7.50 a                     | 0.14 a                 | 0.23 abc        | 0.54 ab     |
|                      |        | Internode 2 | 1.30 ab                                  | 0.06                   | 5.77 ab                    | 0.11 ab                | 0.24 abc        | 0.53 ab     |
|                      | Dough  | Internode 1 | 1.23 ab                                  | 0.06                   | 5.15 b                     | 0.10 b                 | 0.24 abc        | 0.55 ab     |
|                      |        | Internode 2 | 1.29 ab                                  | 0.06                   | 5.07 b                     | 0.10 b                 | 0.24 ac         | 0.53 b      |
|                      | Mature | Internode 1 | 1.14 b                                   | 0.05                   | 6.30 ab                    | 0.12 ab                | 0.17 d          | 0.34 c      |
|                      |        | Internode 2 | 1.06 b                                   | 0.05                   | 5.20 b                     | 0.10 b                 | 0.20 bde        | 0.46 bc     |
| CDC GO               | Milk   | Internode 1 | 1.57 ab                                  | 0.07                   | 6.78 ab                    | 0.13 ab                | 0.23 abcd       | 0.56 b      |
|                      |        | Internode 2 | 1.43 ab                                  | 0.07                   | 6.43 ab                    | 0.12 ab                | 0.22 cde        | 0.56 b      |
|                      | Dough  | Internode 1 | 1.72 ab                                  | 0.08                   | 7.00 ab                    | 0.14 ab                | 0.24 abc        | 0.56 b      |
|                      |        | Internode 2 | 1.76 ab                                  | 0.08                   | 7.17 ab                    | 0.14 ab                | 0.25 abc        | 0.59 ab     |
|                      | Mature | Internode 1 | 1.44 ab                                  | 0.06                   | 5.86 ab                    | 0.11 ab                | 0.26 ab         | 0.61 ab     |
|                      |        | Internode 2 | 1.56 ab                                  | 0.09                   | 6.26 ab                    | 0.12 ab                | 0.25 abc        | 0.74 a      |
| SEM                  |        |             | 0.173                                    | 0.012                  | 0.933                      | 0.019                  | 0.010           | 0.045       |
| Statistical analysis |        |             | -----P value-----                        |                        |                            |                        |                 |             |
| Variety (V)          |        |             | 0.2230                                   | 0.2654                 | 0.5690                     | 0.6200                 | 0.2450          | 0.0644      |
| Stage (S)            |        |             | 0.0060                                   | 0.4633                 | 0.0340                     | 0.0680                 | 0.0001          | 0.7234      |
| Location (L)         |        |             | 0.2860                                   | 0.8898                 | 0.0510                     | 0.1170                 | 0.0790          | 0.0527      |
| V*S                  |        |             | 0.0020                                   | 0.0425                 | 0.0010                     | 0.0020                 | <.0001          | <.0001      |
| V*L                  |        |             | 0.2590                                   | 0.0538                 | 0.0240                     | 0.0800                 | 0.0440          | 0.6116      |
| S*L                  |        |             | 0.0550                                   | 0.0760                 | 0.1440                     | 0.1700                 | 0.3380          | 0.0170      |
| V*S*L                |        |             | 0.6540                                   | 0.3103                 | 0.4720                     | 0.3770                 | 0.0620          | 0.8429      |

SEM = Pooled standard error of means. Means in the same column with different letters are significant different at  $P < 0.05$  level using the Turkey-Kramer procedure



